November 2017

Evaluation of a microsphere-based immunoassay (MIA) in measuring diagnostic and prognostic markers of dengue virus infection

Jason H. Ambrose
University of South Florida, jambrose@mail.usf.edu

Evaluation of a microsphere-based immunoassay (MIA) in measuring diagnostic and prognostic markers of dengue virus infection

by

Jason H. Ambrose, MPH

A dissertation submitted in partial fulfillment
Of the requirements for the degree of
Doctor of Philosophy
with a concentration in Global Communicable Disease
Department of Global Health
College of Public Health
University of South Florida

Major Professor: Thomas Unnasch, PhD
Andrea Bingham, PhD, MSPH
Ricardo Izurieta, MD, MPH, DrPH
Michael Teng, PhD

Date of Approval:
November 13, 2017

Keywords: dengue, MIA, microsphere, hemorrhagic fever, diagnostics

DEDICATION

"We stand upon the shoulders of giants." – paraphrase of Sir Isaac Newton

Firstly, thank you for everything Mom, I love you and miss you every moment……..

This work is, on one hand, dedicated to everyone and anyone that helped me through this process. The number of individuals that have put me back on track, whether consciously or not, whether I've met you or not, is legion. Thank you. On the other hand, this work is also dedicated to the countless individuals affected by infectious diseases, particularly of those described within the body of this text. It is my hope and great endeavor that the fruits of my labor (past, present, and future) have a positive effect in gaining a better understanding of that which plagues us. Ultimately, investigations such as this may lead to a watershed moment in humanity where we collectively gain the universal wherewithal, in addition to realizing our absolute moral obligation, to successfully manage, curtail, prevent, and ultimately eliminate infectious disease entities for the betterment of us all.

I'd also like to personally dedicate this work to my family members that are no longer physically with us: my parents: Lorraine Baumes-Ambrose and Henry Ambrose, Jr., my grandparents: George and Agnes Baumès, Henry Ambrose, Sr., and my uncle Paul Baumès. I hope somewhere you're smiling.

ACKNOWLEDGEMENTS

Support in any endeavor comes from many sources, such as academic, material, and moral. First and foremost, I'd like to thank my current committee members for their support and expertise: Drs. Thomas R. Unnasch, Ricardo Izurieta, Andrea Bingham, and Micheal Teng, in addition to past members Drs. Lillian M. Stark, Azliyati Azizan, Richard 'Ran' Nisbett and Frederick Heinzel. I'd also be remiss to not mention and extend my lasting gratitude to everyone that had some substantial input in the project: Drs. Christy Ottendorfer, Alberto van Olphen, Shamala Devi Sekaran, Wang Seok Mui, Andrew Falconar, Holly Hughes, and Aimee Signarovitz; along with Jazmine Mateus, Priscila Iwakawa, Rita Judge, Maribel Castaneda, Logan Haller, Heidi Hernandez, Kelly Fitzpatrick, Cindy Bucher, Kathy Zahn, Donna Hardy, Cheong Huey Chiat, Ben Klekamp, Ann Mitulinsky, Paul Mackley, Elisabeth Lim, Frantz Jean Louis, Tatiana Gardellini, Jane Barkan, Lea Heberlein-Larson, and Natalie Wright. Your collective assistance and advice were indispensable.

Other acknowledgements include Drs. Aparna Tatavarthy, Donna Haiduvan, Andrew Cannons, Boo Kwa, Rick France, Jameela Sathar, Michael Steele, Ken Pidcock, Paul Young, Eva Harris, Paul Adams, Barbara King, and Kay Perrin; along with Debbie Chapman, Ellen Kent, Jessica Grossman, Eddie Tensley, Susan Lukas, Alan Zellner, and Rick Gaetano. I'd also like to thank the rest of my colleagues and co-workers at the DOH Tampa lab: Brandon Ebanks, Sarah York, and Tony Williams come particularly to mind.

Ultimately, however, I don't think I'd have been able to complete this arduous task without the support of my friends and family. To my friends, in particular Brian Dorshefski, Brian Rohr, Jon Trosky, Steve DiPippa, and Alessandra Rodriguez. Caesar and Roo, my min pins that have been with me nearly daily, on both the good and the bad days. Finally, again, to my parents Henry and Lorraine Ambrose, my sister and her family, Shevaun and Rich Olshefski and their son Zachary, my grandmother Lorenzina Ambrose, along with my entire family…….from the bottom of my heart, thank you

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

α - alpha
~ - approximate
@ - at
β - beta
°C - degrees Celsius
(/) - divisor: may refer to the mathematical function (*i.e.* fraction) or to either/or situations
(=) - equal to
γ - gamma
µg - microgram
µL - microliter
(-) - negative/minus/below
% - percent
(+) - positive/plus/above
® - registered trademark
ʎ - (lambda) wavelength
3-D - 3-dimensional
3' - 3 prime coding region of genome
5' - 5 prime coding region of genome
AA or aa - amino acid
Ab - antibody
A/Abs - absorbance
ADE - antibody-dependent enhancement
Ag - antigen
arbo - arthropod-borne
ATCC - American Type Culture Collection
appx - approximately
c - viral capsid protein
CDC - Centers for Disease Control and Prevention
cm - centimeter
CNS - central nervous system
CO_2 - carbon dioxide (gaseous)
COOH - carboxyl group
CPE - cytopathic effect
CSF - cerebrospinal fluid
C_T - cycle threshold
d - day
DC-SIGN - dendritic cell-specific ICAM-3 grabbing non-integrin
DENV/DENV1-4 - dengue virus/(*as a group*)
DEN# - dengue virus (*# denotes serotype*)
DHF - dengue hemorrhagic fever
DNA - deoxyribonucleic acid
DSS - dengue shock syndrome
EBSS - Earle's balanced salt solution
EBM - endothelial cell basal medium
EC - endothelial cell
EDTA **-** ethylenediaminetetraacetic acid
EGM - endothelial cell growth medium
ELISA - enzyme-linked immunosorbent assay
env - viral envelope protein
FBS - fetal bovine serum
Fc_x - immunoglobulin functional class (*constant region*)

Fc_xR - immunoglobulin functional class (*constant region*) receptor
FDA - (United States) Food and Drug Administration
FGF - fibroblast growth factor
FLDOH-BPHL - Florida Department of Health, Bureau of Public Health Laboratories
g - gram
GM-CSF - granulocyte-macrophage colony stimulating factor
gp - glycoprotein
h - hour
h - *as a prefix*, denotes human
H_2O - water
HAI/HIA - hemagglutination-inhibition assay (*alt. form HIA*)
hEGF - human endothelial growth factor
HEPES - 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HIA - hemagglutination-inhibition assay (*alt. form HAI*)
HIV - human immunodeficiency virus
HLA - human leukocyte-associated antigen
HMEM - Hank's minimal essential medium
HPMEC - human pulmonary microvascular endothelial cells
IBC - institutional biosafety committee
ICAM-# - intercellular adhesion molecule (# *denotes specific type*)
ICT - immunochromatographic test
ICTV - International Committee on Taxonomy of Viruses
IFN-x - interferon (-x *denotes Greek letter for specific type*)
IGx - immunoglobulin (x *denotes specific type*)
IL-# - interleukin (# denotes specific type)
IP-# - interferon-γ inducible protein (# *denotes specific type*)
JEV - Japanese encephalitis virus
L - liter
M - molar
mAb - monoclonal antibody
MAC - membrane attack complex
MCP-# - monocyte chemoattractant protein (# *denotes specific type*)
M-CSF - macrophage colony stimulating factor
MEM **-** minimal essential medium
mg - milligram
MHC - major histocompatability complex
MIA - microsphere-based immunoassay
min - minute
MIP-# - macrophage inflammatory protein (# *denotes specific type*)
mL - milliliter
mm - millimeter
mM - millimolar
MOI - multiplicity of infection
NC - non-coding region (*synonymous with UTR, untranslated region*)
NaCl - sodium chloride
NCBI - National Center for Biotechnology Information
NCS - newborn calf serum
NEAA - non-essential amino acids
neg - negative
NF-κB - nuclear factor kappa enhancer binding protein
ng - nanogram
nm - nanometer
nt - nucleotide
NO - nitric oxide
NOS - nitric oxide synthase
NS# - viral non-structural protein (# *denotes specific type*)

qRT-PCR - quantitative reverse transcription-polymerase chain reaction
PAHO - Pan-American Health Organization
PBMC - peripheral blood mononuclear cell
PCR - polymerase chain reaction
PDGF - platelet-derived growth factor
PECAM - platelet/endothelial cell adhesion molecule
pfu - plaque forming unit
pg - picogram
p.i. - post-inoculation
prM/M - viral premembrane/membrane protein
pos - positive
r - *as a prefix*, recombinant
RANTES - regulated upon activation normally t-cell expressed and secreted
rcf - rotational centrifugal force
RNA - ribonucleic acid
RNase - ribonuclease
RNasin - ribonuclease inhibitor
rpm - rotations per minute
RT - reverse transcription
RT-PCR - reverse transcription-polymerase chain reaction
s/sec - second
SECEBT - Southeast Center for Emerging Biologic Threats
SNP or snp - single nucleotide polymorphism
SLEV - St. Louis encephalitis virus
TAE - tris-acetate-ethylenediaminetetraacetic acid (EDTA)
Taq - *Themophilus aquaticus*
T_C - cytotoxic T-cell
T_H - T-helper cell
™ - trademark
TNF-x - tumor necrosis factor (-x *denotes specific type*)
USA - United States of America
USF-COPH - University of South Florida, College of Public Health
USNIH - United States National Insitutes of Health
UTR - untranslated region (*synonymous with NC, non-coding region*)
UV - ultraviolet light
v - *as a prefix*, viral
VCAM - vascular cell adhesion molecule
VEGF - vascular endothelial cell growth factor
w/ - with
WHO - World Health Organization
WNV - West Nile virus
x - multiplier, mathematical function
x g - times the force of gravity, Earth (*synonymous with rcf, or, rotational centrifugal force*)
YVF - Yellow fever virus

Note: Separate lists for amino acid and nucleotide abbreviations appear in A.2. and A.3.

ABSTRACT

Infections with dengue viruses (DENV) constitute both a global problem as well as locally in Florida. DENV comprise four distinct serotypes of single-stranded RNA viruses and belong to the family *Flaviviridae*. DENV are among the most medically important arboviruses in the world and cases may currently exceed 400 million per annum. Additionally, dengue established its first recorded endemic transmission cycle in the state of Florida in over a half century, first within the Florida Keys during 2009-10 followed by an unrelated outbreak in Martin County in 2013. The clinical profile of DENV infections ranges from a mild febrile illness to severe illness including hemorrhaging and/or shock, occasionally leading to death. Asymptomatic and mild cases also play a role in maintaining transmission cycles. The early diagnosis and management of patients at the clinical level have both proven to be major obstacles in the control of DENV and are important at both the individual and community levels. Individually, the proper management of patients that will progress to severe illness demands that they are identified in order to receive supportive treatment and mitigate associated morbidity and mortality. At the community level, early diagnosis may reduce transmission by limiting the possibility of vector contact with viremic individuals. Early diagnosis is dependent on the detection of viral markers, while a number of host factors may inform prognosis. The microsphere-based immunoassay (MIA) is capable of detecting up to 100 different targets in a single sample and therefore would be useful as a single assay for determining both. This study attempted to develop a diagnostic and prognostic MIA using the DENV NS1 glycoprotein and 5 host markers as targets. For the purposes of DENV NS1 detection in MIA, a set of monoclonal antibodies (mAbs) were subjected to immunoprecipitation and/or Western blot analysis in order to determine immunoreactivity. Two mAbs, 3A5.4 and 3D1.4, were chosen for use in MIA as a capture antibody and a detection antibody, respectively, and the results compared to a commercially available DENV NS1 ELISA. The 5 markers chosen for MIA trials included GM-CSF, IFN-γ, IP-10, IL-10, and MCP-1 and were selected from a panel of 27 markers screened initially in two *in vitro* models of DENV infection in

addition to serum samples. The two cell lines investigated were HPMEC ST1.6R and u937 as both are thought to play important roles in models of DENV pathogenesis. The results of the DENV NS1 detection MIA were initially promising but were ultimately unsuccessful. When measuring host markers in the MIA, results pointed towards certain profiles that may be of future use. IL-10 was found to be elevated in a statistically significant manner in DENV qRT-PCR+ samples ($p=0.035$) when compared to negative sera. MCP-1 elevation was found to be of borderline significance ($p=0.058$). Other potential markers based on the results reported here include IP-10, IL-6, IL-8, VEGF, and RANTES. The ultimate goal of measuring host markers is to gain the ability to differentiate patients that will progress to severe illness from those that will recover. In conclusion, despite the failure of the MIA to detect DENV NS1 in human sera, our results in determining host markers and developments leading to successful DENV NS1 detection ELISAs elsewhere lead us to believe that this approach remains promising. Major drawbacks of this study included the lack of samples from patients that experienced severe DENV illness as a comparative group in addition to small sample sizes. Future goals should include determining the reasons for the failure of the MIA in detecting DENV NS1, selecting a panel of appropriate markers to differentiate non-severe from severe cases of DENV prior to progression, and optimizing the inclusion of these markers to an appropriate number.

CHAPTER 1. LITERATURE REVIEW, STATEMENT OF THE PROBLEM, AND SPECIFIC AIMS

1.1. Literature Review

1.1.1. Introduction - the Flaviviruses

The family *Flaviviridae* includes 3 genera: Flavivirus, Hepacivirus, and Pestivirus. Within the flaviviruses, the literature mentions that there are now over seventy recognized members of the genus. This is a somewhat reduced number from that historically recognized, as the family *Flaviviridae* has recently been separated from the *Togaviridae*. (Petersen and Barrett, 2009) The formal separation occurred after 1980 due to the work of Calisher and others (Calisher *et al*., 1989). This number is slowly increasing due both to previously unrecognized viruses emerging in addition to divergent evolution of extant species. It should be noted that as of 2014 the ICTV database lists 53 species (ICTV, 2014) while in contrast there are 116 listed in the NCBI taxonomy database (with DENV1-4 as a single species) (NCBI, 2014). Regardless, nearly all species within the genus Flavivirus are maintained in a transmission cycle between arthropod and vertebrate hosts, while many are known to affect humans. The name flavivirus itself is derived from that of the prototype virus of the family, the yellow fever virus (YFV). Yellow fever has been recognized as a disease entity as far back as 1647-1648 when it first caused epidemics in the New World (Marr, 2013; Staples and Monath, 2008) while dengue has been described specifically since 1827 (Gibbons *et al*., 2012; Horlbeck, 1896; Thomas, 1880).

Flaviviruses encode a genome of approximately 10,000 nucleotides in the form of a positive-sensed, single-stranded RNA. The genome is arranged in a 5'-3' manner encoding 10 proteins (3 structural, 7 non-structural) in a single open-reading frame (ORF) and is capped on both ends by functional non-coding regions. Genomic organization is the primary reason for their separation from alphaviruses (*Togaviridae*), as flaviviruses lack a 3' poly-A tail and as opposed to alphaviruses, their structural proteins are located upstream (5') with regards to the non-structural proteins. The 3 structural

proteins of flaviviruses are located at the 5' end of the ORF and include, in order, the capsid (C), pre-membrane/membrane (prM/M), and envelope (env or E) proteins. (Petersen and Barrett, 2009)

The envelope protein is considered to contain the dominant immunogenic epitope responsible for generating what are thought to be the most effective neutralizing antibodies in humans. Further downstream, the genome encodes 7 non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and the viral RNA-dependent RNA polymerase, NS5. Historically, flaviviruses have been classified into as many as 8 complexes according to antigenic responses within neutralization tests. (Petersen and Barrett, 2009) Genetic analyses based upon arguably the most diversified viral protein (env) and the most conserved (NS5) have placed flaviviruses in one of four phylogenetic clades: Culex, Aedes, Tick-borne, and Rio Bravo. The Culex clade includes viruses that are primarily vectored by *Culex spp*. mosquitoes and usually cause encephalitic disease in incidental or dead-end hosts, such as humans. These viruses include West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Kunjin virus, and Japanese encephalitis virus (JEV). The Aedes clade viruses are most often transmitted by *Aedes spp*. mosquitoes, have the capacity to cause hemorrhagic fever in humans, and contains the prototypical flavivirus, yellow fever virus (YFV). This clade additionally counts dengue virus (DENV) serotypes 1-4 and Zika virus (ZIKV) among its members. The third clade contains those flaviviruses vectored by ticks and includes tick-borne encephalitis virus, Kyasanur Forest disease virus, louping ill virus, Powassan virus, and Omsk hemorrhagic fever virus. The fourth and final clade of flaviviruses determined by this analysis contains viruses with no known vectors and the group is named after its prototype virus, Rio Bravo virus. Each of these clades is further grouped to both geographic location (Old World vs. New World) and vertebrate host (*i.e.* birds/rodents/humans). (Gaunt *et al*., 2001)

1.1.2. Medically Important Flaviviruses Occurring in Florida

SLEV, WNV, and DENV are all thought to be present in FL as of 2014. WNV is known throughout the state, SLEV is traditionally thought to be absent from southern FL but has recently been detected as far south as Miami (FLDOH-BPHL-Tampa, *unpublished data*). These two viruses are thought to overwinter in various hosts in Florida but each may currently be present only seasonally in concert with

migratory bird populations (Rappole *et al*., 2000; Dunbar *et al*., 1998, Day *et al*., 1996). These migratory birds may re-introduce WNV and/or SLEV in locales where they may have gone extinct, and although unlikely, some years this 'local extinction' may arguably encompass the entirety of the state, especially in recent years for SLEV. This absence of SLEV activity in Florida in recent years may be due to displacement of the virus by WNV transmission, similar to what has been suggested in southeastern California (Ottendorfer *et al*., 2009; Reisen *et al*., 2008). DENV has established limited transmission for the first time in decades within Florida, and has caused outbreaks in Key West and Martin County. In addition to these outbreaks, patients reporting no travel history have been diagnosed with dengue from numerous counties in Florida during the last three years, including Broward, Hillsborough, Miami-Dade, Monroe, Orange, and Palm Beach with no record of its sustained transmission (FLDOH, 2014).

The distribution of WNV/SLEV and DENV do not generally overlap as they exhibit different ecologies. WNV and SLEV exhibit a zoonotic ecological pattern, whereas it is well established that DENV transmission primarily occurs in an urban setting involving only humans and mosquitoes. In theory, the different ecologies of the three viruses should allow Florida to support endemic transmission of each within the state. While the ecologies of SLEV and WNV are very similar, they may differ enough in certain reservoir hosts to escape competitive exclusion (*i.e.* birds/reptiles for WNV compared to birds/rodents for SLEV). (Petersen and Barrett, 2009) Additionally, these two zoonotic viruses would likely have little impact on the ecology of the primarily human-adapted, urban-transmitted dengue virus. This means that Floridians may be at risk for acquiring infections with any, or all, of these viruses. Additionally, if antibody-dependent enhancement of infection (ADE) occurs among different species of flaviviruses rather than just among different serotypes of DENV, Floridians and visitors to the state may be at increased risk of severe illness following a secondary infection of a heterospecific virus. This phenomenon of ADE is not known to occur outside of sequential infection with heterologous serotypes of dengue, however, circumstantial evidence supports that it may have occurred in at least one instance. A Florida man infected with WNV succumbed to a fatal hemorrhagic fever that also had previous exposure to DENV (Paddock *et al*., 2006). Furthermore, circulation of multiple flaviviruses in FL requires that functioning diagnostic capabilities be present in order to differentially detect WNV, SLEV, and DENV. Additionally, with the onset of the Zika virus pandemic and its subsequent introduction and local transmission in Florida, the epidemiological

picture for flaviviruses in Florida has once again changed dramatically. One thousand fifteen (1115) travel related-cases and 218 autochthonous cases were detected within the state during 2016 since its hypothesized introduction that spring. However, only 80 travel-related cases and 1 locally acquired case have been observed through October of 2017. The ecology of ZIKV is similar to that of DENV and it remains to be seen how its introduction may affect both ZIKV and DENV transmission in Florida. (CDC, 2017; Grubaugh *et* al., 2017; Likos *et al*., 2016) Correct diagnosis of the offending viral agent is important for managing patients in the sense of supportive care and certainly impacts vector-control measures with regards to targeting proper vector habitats and mitigating potential outbreaks. Establishing these capabilities takes expertise, time, research, and funding.

1.1.3. Dengue Virus (DENV)

1.1.3.1. DENV - Introduction

Infection with dengue virus continues to be a worldwide threat to public health both in human and economic costs. In 2017, dengue virus is arguably still the most significant arthropod-borne (arbo-) viral illness. The virus and its mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, continue to encroach upon new territory. In 2009-2010, dengue virus established its first recorded endemic transmission cycle in the state of Florida in over a half century, first within the Florida Keys during 2009-2010, followed by an unrelated outbreak in Martin County in 2013 (Florida Health, 2015; Munoz-Jordan *et al*., 2013; Anez *et al*., 2012). Other recent outbreaks have occurred in the United States in Hawai'i (2002 and 2015) and Texas (2002 and 2013) excluding outlying territories such as Puerto Rico (Johnson *et al*., 2016; Thomas *et al*., 2016; Petersen and Barrett, 2009). Worldwide, an estimated 50-100 million infections occur annually with an at risk global population of 2.5 billion according to WHO figures. Approximately 50,000 of these patients will progress to severe illness and require hospitalization, around 1250 of these patients will die. (WHO, 2011) Other models suggest the current true burden of dengue at around 400 million annual cases where approximately 100 million seek medical attention with a total of 4 billion at risk (Gulland, 2013).

Lack of therapeutic treatment options coupled with a vaccine in its initial stages of use in parts of Asia and Latin America (Wichmann *et al*., 2017), still leaves proper clinical management as the only primary option for reducing morbidity and mortality associated with dengue. Infection with DENV leads to three stages of illness, the acute/febrile phase, the critical phase, followed by either recovery or worsening to severe illness. Severe illness may include any or all of the following: hemorrhage, shock, and/or death, but is primarily characterized by plasma leakage due to an increase in vascular permeability. The febrile phase begins abruptly from 2-7 days after infection with non-specific symptoms. Most often, when patients present at the clinical level during this phase, those who may progress to severe illness and those that will experience uncomplicated illness are indistinguishable. The beginning of the critical phase usually coincides with defervescence on days 3-7 of illness, and lasts 24-48 hours. It is at this stage where 'warning signs' for development of severe illness most often become apparent and certain patient criteria are evaluated for admitting and managing patients, ranging from presence of fever to evidence of plasma leakage. At any stage prior to this, a diagnostic tool yet to be developed that could identify and distinguish patients according to severity would be of tremendous value. (WHO, 2009)

1.1.3.2. DENV - History

While dengue is not a disease described in antiquity, *per se*, the illness itself was described long before the causative viral agent was identified. Anecdotal evidence suggests that the disease was described in China as far back as 992 as 'water-poison' associated with 'biting flies' as related in Halstead, 2008 and Gubler, 1998 from a 1979 report by Nobuchi. Dengue was identified in Philadelphia during the late 18th century (Rush, 1879), and the global picture changed after conflict in Asia due to World War II (Brathwaite Dick *et al*., 2012; Gibbons *et al*., 2012; Halstead, 2008).

The term dengue itself proves something of an etymological problem. Break-bone fever predates the usage of dengue and it is believed that the word is a Spanish corruption or translation of 'dandy fever': the term given to the illness by displaced Africans in St. Thomas (likely slaves) circa. 1827. In turn, the Spanish coined the term 'el dengue' or 'the afflicted' to describe the illness only one year later in Havana. 'Dengue' was subsequently accepted by the Royal College of Physicians and Surgeons in London as

official nomenclature and it was further proposed that the term be Latinized to *denguis*, though this never panned out. (Thomas, 1880)

1.1.3.3. DENV - Structure and Biology

Dengue disease is caused by one of four different dengue virus serotypes of small RNA viruses in the family *Flaviviridae*. Each of these four serotypes are thought to have emerged from a common ancestor, the recently discovered putative fifth serotype in Sarawak, Malaysia seems to support this theory (Schnirring, 2013). Having emerged from a common lineage would seem to imply conservation of common epitopes and this can be evidenced through the very narrow epidemiological picture dengue viruses present as is currently understood (Chen and Vasilakis, 2011; Vasilakis and Weaver, 2008).

The 5' and 3' ends of the DENV genome contain untranslated regions (UTRs) that vary in size according to serotype. These UTRs form secondary structures that are important in viral replication and involve both RNA-RNA and RNA-protein interactions. Both are highly conserved among DENV serotypes, the 5' region contains between 95 and 101 nucleotides while the 3' UTR of DEN1 contains 470 bases and DEN2, -3, and -4 about 385 (Gamarnik, 2010). Although highly conserved, genetic differences do arise, for instance in the DEN3 3' UTR, that are thought to be meaningful and may have an impact on virulence (Goncalves de Castro *et al*., 2013).

The DENV genome's open reading frame encodes, first, the three structural proteins, C, prM/M, and env, followed by 7 non-structural proteins (Fig. 1.1). It is translated as a single polyprotein that is processed and modified post-translationally. The first of these genes, C, encodes for the viral capsid, a 113-amino acid protein, and together make up the nucleocapsid surrounding the viral genome. The capsid also interacts with both UTRs. The prM/M gene encodes for membrane proteins that exist in two forms, thus the pre-membrane (prM) and membrane (M) designations. The prM is seen in immature virions and is thought to be essential for proper folding of the env protein via chaperoning mechanisms. The M protein is incorporated along with the env protein in mature virions to form, along with a host-derived lipid bilayer, the viral envelope. (Petersen and Barrett, 2009)

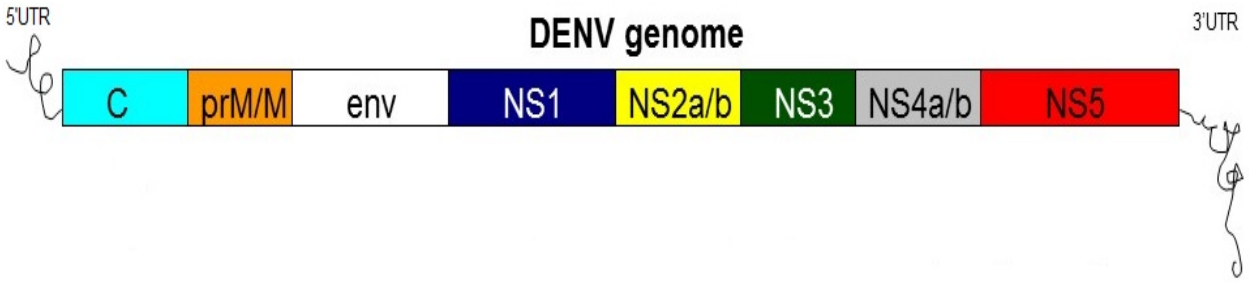

Fig. 1.1: Representation of DENV genome by gene loci. The genome of dengue viruses, like that of all flaviviruses, is arranged in a 5'-3' manner and encodes, first, genes for the three structural proteins (C, prM/M, and env) followed by 7 non-structural proteins (NS1, NS2a/b, NS3, NS4a/b, and NS5). It also contains functional untranslated regions (UTRs) at each end of the genome (5' and 3'). (*Author's image*)

The envelope protein of DENV itself functions primarily as the major factor in the process of infecting host cells and is the major antigenic determinant of the host immune response. In DEN1, -2, and -4 the env protein consists of 495 amino acids and in DEN3, 493 (Beasley and Barrett, 2010). The env protein itself contains three functional domains (I, II, and III) composed of β-barrels and exhibits approximately 40% homology in amino acid identity across flaviviruses (Perera *et al*., 2008). Domain I is located centrally and serves as a hinge region for the other two domains. Domain II contains two loop structures and these loops function together as a site where fusion between the virus envelope and host-cell membranes occurs. This second domain is very highly conserved among flaviviruses. Not surprisingly, antibodies generated against these loop regions are broadly cross-reactive because of this conservation. Domain III is described as an immunoglobulin-like motif and contains four loops on its lateral ridge. Domain III contains both conserved and species-specific sequences, thus likewise, cross-reactive and highly specific antibodies are generated against it. However, this region is where the most highly specific, strongly neutralizing antibodies are generated against DENV in humans. (Goncalvez *et al*, 2010; Petersen and Barrett, 2009; Perera *et al*., 2008; Seligman *et al*, 2008)

There are 7 non-structural proteins that are encoded by the DENV genome in a 5' to 3' direction: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. NS3 proteins consist of 618-623 amino acids and it functions as the viral protease responsible for processing of the polyprotein. It also plays a role in viral replication as NS3 has nucleotide triphosphatase, RNA 5' triphosphatase, and helicase activities. It is also the most conserved protein among the DENV group. The NS5 protein serves as the virus' RNA-dependent RNA polymerase, essential for replication, and consists of 900-905 amino acids. Potential

methyltransferase motifs are found at the N-terminal region. NS2A, NS2B, NS4A, and NS4B are small hydrophobic proteins and may play roles as co-factors during viral replication and in pathogenesis. (Beasley and Barrett, 2010; Miller *et al*. 2010)

The NS1 protein of DENV is found both intracellularly as well as in a soluble form (sNS1) secreted from infected mammalian host cells and is of enigmatic function. Its immature form is that of a monomer that can range from about 40-49kD and consists of either 353 or 354 amino acids. It readily forms homodimers of approximately 80kD that are heat labile and usually associated with the surface of infected cells (Beasley and Barrett, 2010; Falconar and Young, 1991). From there, the major oligomeric form of soluble NS1 is thought to be a hexamer of around 300kD. The hexamer consists of 3 dimeric units that are non-covalently bound and are less stable than NS1 dimers (Gutsche *et al*., 2011; Flamand *et al*., 1999). DENV NS1 hexamers consist of a symmetric 'barrel-shape' that carries lipid cargo in its central core, whereas dimer subunits are constructed around a central β-sheet domain. Monomers consist of three domains: a small β-roll dimerization domain (aa 1-29), a 'wing domain' (aa 30-180), and the predominant feature of the protein, a central β-ladder (aa 181-352). The 'wing domain' is surface exposed and thought to be a major antigenic site of the protein. In fact, while this 'wing domain' is highly conserved and cross-reactive with host proteins, mAbs directed at this site have shown to have a protective effect in DENV infections. 'Wing-domain' derived protein subunits elict a strong enough immune respsonse to be considered as vaccine candidates. Figure 1.2 illustrates the 3-D structure of a DENV NS1 hexamer comprised of its constituent dimer subunits, where the lobes or 'wing domain' is the dominant antigenic epitope. (Lai *et al*. 2017; Yap *et al*. 2017; Akey *et al*., 2014; Muller *et al*., 2012; Gutsche *et al*. 2011; Liu *et al*. 2011) Secretion of the protein apparently does not abrogate replication, however its presence is required for successful virion assembly and the 'wing domain' is thought to play a critical role (Scaturro *et al*. 2015). DENV NS1 is variably glycosylated at two conserved sites, Asn130 and Asn207. The former site incorporates high complex glycans while the latter is found to add high mannose glycans. DEN2 mutants lacking glycosylation at Asn207 show decreased stability but this mutation does not abrogate dimerization. The presence of glycans at this site are also thought to modulate secretion of NS1. Glycosylation at Asn130 is thought to help in stabilizing hexamers of NS1 and is required for interactions with proteins of the complement system. (Akey *et al*., 2014; Avirutnan,

2011; Somnuke *et al*., 2011; Pryor and Wright, 1994) It was previously thought that NS1 was not secreted from mosquito cells, or at least mosquito-derived cell cultures (Alcon *et al*., 2002; Flamand *et al*., 1999) but new evidence suggests that the protein is, in fact, secreted in vector mosquitoes in a caveolin-dependent pathway that alters its post-translational modifications (Alcala *et al*. 2017).

This protein (NS1) is also thought to modulate DENV disease severity for a variety of reasons. Secreted NS1 is thought to interact with uninfected endothelial cells, preferentially those of the microvasculature of the lungs via heparin sulfate and chondroitin sulfate E, thus leading to capillary leakage (Avitrutnan *et al*., 2007). Antibodies against NS1 are believed to cross react with human fibrinogen and platelets, both thought to be critical factors in DENV illness, especially severe manifestations (Arya *et* al., 2011; Honda *et* al., 2009; Falconar, 2007).

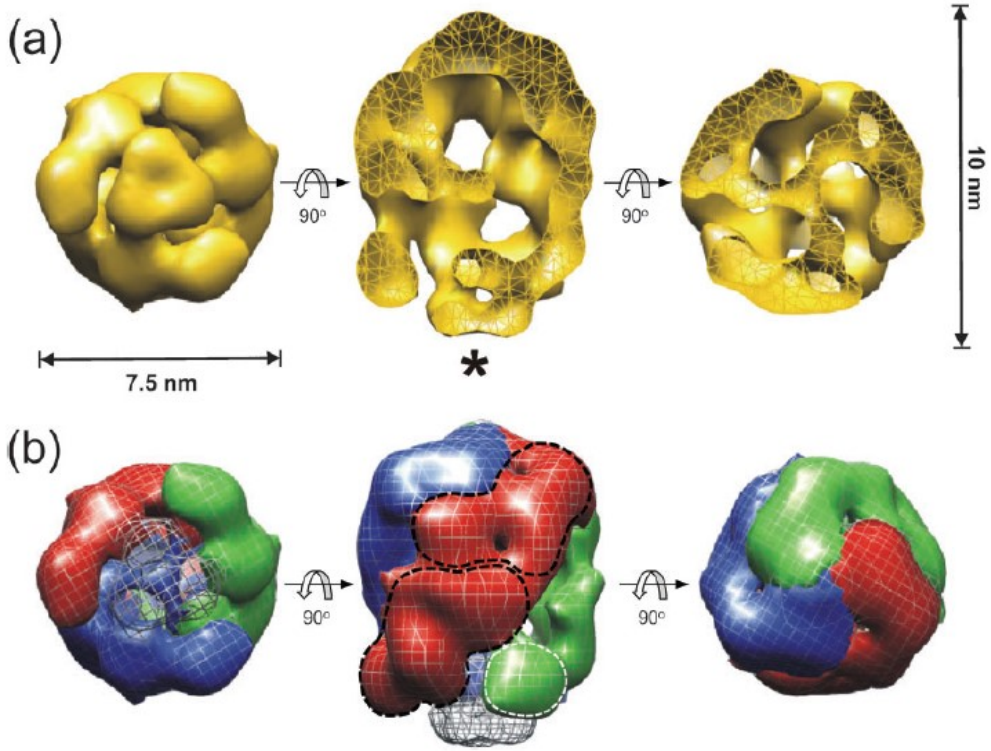

Fig. 1.2: Three dimensional (3-D) rendering of DENV NS1 protein. This figure illustrates the 3-D rendering of DENV NS1 hexamer. In the bottom image, red, blue, and green denote dimer sub-units, the lobes of each ('wing domain') are thought to be major antigenic sites for DENV NS1. *Image reprinted with permission courtesy of Paul Young*. (*From* Muller *et al*., 2012)

1.1.3.4. DENV - Virus/Vector Relationships

DENV is an arbovirus and as such is transmitted to humans via mosquitoes. However, unlike other arboviral flaviviruses, such as WNV and SLEV, that are zoonotic in nature and where humans are incidental hosts, *i.e.*, not capable of developing viremia sufficient to infect a vector, the ecology of dengue virus depends on that of humans and vector mosquitoes and both are required for establishing transmission cycles. Therefore, the number of mosquito species serving as suitable vectors for DENV tends to be more restrictive than that of other flaviviruses, and it is much more like YFV in this respect. Due to the limited range of adult Aedes females, or their reluctance to search outside of their narrow domestic habitats, the spread of DENV within a community at large is thought to be more reliant on the movement of people. (WHO, 2009) Transmission of DENV normally occurs in humans via the bite of an infected mosquito when the virus is injected after secretion from the mosquito salivary glands. The extrinsic incubation period, where the virus multiplies within the vector, is typically between 10-14 days. This type of transmission either requires the female mosquito, infectious after a prior blood meal, biting a second human, or vertical transmission of the virus to the mosquito offspring. (Halstead, 2008)

The primary vector of dengue virus is *Aedes aegypti*; the ecology of this mosquito is closely tied to that of human populations. *Ae. aegypti*'s habitat is thought to originally have been in Africa and its spread to the Americas was concomitant with that of the African slave trade. Initially restricted to Caribbean islands and port cites on the mainland, the species greatly increased its range to include the interiors of North and South America during the early 1900s, where conditions were suitable. Upon the initiation of the vector eradication program to combat yellow fever, it was nearly extirpated from the Western Hemisphere. (Soper, 1967) After the crumbling of the vector-eradication program of the 1950s and 1960s, *Ae. aegypti* made a remarkable comeback. Today it is present across the entire region including parts of N. America, encompassing a range rivalling its once former peak. Both social and climatological factors contribute to the distribution of *Ae. aegypti*, as evidenced throughout Latin America and the Caribbean. (PAHO, 1997) *Ae. aegypti* preferentially breeds in human-generated water containers and feeds almost exclusively on humans during the daytime. It has been described as a

furtive feeder and may seek multiple hosts during the course of a blood meal, introducing the possibility of mechanical transmission of DENV. (Ooi and Gubler, 2010; Halstead, 2008; Christophers, 1960)

To a lesser extent, *Ae. albopictus*, the Asian Tiger mosquito, is also implicated as a vector of human transmission of DENV. The species originated in SE Asia in an arboreal setting but eventually adapted to urban conditions, much like *Ae. aegypti*. For this reason, *i.e.* the species' more recent domestication, it tends to be a more generalized feeder (not as strictly anthropophilic). In recent decades, it has greatly expanded its range, mostly via anthropogenic methods of travel and is now found on every permanently-inhabited continent by humans. The two species have been known to co-exist in certain locales but, on the other hand, *Ae. albopictus* has been also found to displace populations of *Ae. aegypti*. Both situations are due in large part to the adaptation of *Ae. albopictus* to the preferred breeding sites of *Ae. aegypti*. (Ooi and Gubler, 2010) *Ae. albopictus* is also a diurnal feeder but has been found to be more aggressive in feeding than *Ae. aegypti*. The former is less likely to seek multiple hosts during a blood meal due to interrupted feeding, hence its colloquial name, "Tiger" (Ooi and Gubler, 2010). Furthermore, *Ae. albopictus* has demonstrated greater tolerance to colder climates and may therefore exhibit a greater maximum range than that of *Ae. aegypti*. While considered a secondary vector, *Ae. albopictus* is nonetheless a competent and important vector involving the transmission of DENV. Anecdotal evidence suggests that *Ae. albopictus* may have contributed in the transmission of DENV to humans to at least the mid-19th century while within its home range of SE Asia. (Paupy *et al*., 2009)

Aedes species other than *Ae. aegypti* and *albopictus* serve as vectors of DENV in sylvatic cycles due to their dominant roles in vector-host relationships in these settings as compared to the former two urban mosquito species. The collective habitats, breeding grounds, and behavior of sylvatic Aedes mosquitoes are thought to resemble that which has been theorized for both of the above species prior to their respective domestication. These species include, among others, *Ae. luteocephalus*, *Ae. furcifer*, *Ae. taylori* (in Africa), Aedes species in the group 'niveus' (in SE Asia), *Ae. polynesiensis* (on Polynesian islands), and *Ae. triseriatus* and *Ae. mediovittatus* (in the Caribbean). (Barrera *et al*., 2012; Halstead, 2008)

1.1.3.5. DENV - Epidemiology and Ecology

The global emergence of DENV is thought to have been concomitant with the aftermath of World War 2 (Brathwaite Dick *et al*., 2012; Gibbons *et al*., 2012; Halstead, 2008). The number of cases detected has risen from less than 1000 from 1955-59 to approximately 1 million between 2000 and 2007. As of 2015, an estimated 50-100 million infections with DENV occur annually. The total at risk global population is approximately 2.5-4 billion people living within the tropics and sub-tropics. Some have suggested, based on mathematical models, that the current annual burden of dengue contains a large degree of error, with a range of about 75-200 million cases (Bhatt, 2013). Until very recently the only WHO region not reporting local transmission of DENV was the European region. Over the past few years prior to 2014, autochthonous cases have been detected in countries such as France and Germany, with outbreaks occurring in Spain and Portugal, likely originating in Madeira (WHO, 2012).

Globally, nearly 70% (1.8 billion) of at risk populations reside within the WHO regions of SE Asia and the Western Pacific, and most of the remaining burden occurs in C. and S. America and the Caribbean. The Pan-American Health Organization (PAHO) subdivides the South American region; where within the Southern Cone region alone approximately 98.5% of cases are reported from Brazil. Outside of the Southern Cone, within the Andean region, 81% of DENV cases are reported from Colombia and Venezuela, a region showing many similarities to that of most of Brazil (WHO, 2009). These two countries also possess both the overland gateways to S. America as well as important ports. In 2008 the combined region of the Caribbean, Mexico, C, and S. America reported 908,926 cases of dengue. (PAHO, 2011) In North America prior to 2009, indeed even after, the areas most at risk of local transmission are those bordering endemic regions and those experiencing high levels of traffic between endemic regions. For instance, areas where local transmission have occurred include Hawai'i (distant to the mainland in the 'Western Pacific') along the border between Texas and Mexico, and in Florida (adjacent to the Caribbean region). These areas are all connected to endemic regions and experience high-levels of movement of people between endemic and non-endemic areas. (Munoz-Jordan, *et al*., 2013; Anez *et al*., 2012; Petersen and Barrett, 2009; Rawlings *et al*., 1998)

As of 2008 most countries in Latin America and the Caribbean reported at least two circulating serotypes of dengue. Among these countries are Brazil, Mexico, and Puerto Rico (USA): places that are well documented to have issues with dengue transmission. By 2013 most countries within contiguous Central and South America have indeed reported all four circulating (Fig. 1.3) (PAHO, 2011). It should be noted that generally the burden of dengue is lower in the Caribbean than in contiguous Latin America. Economic disparity between island nations of the Caribbean also seems to play a role as the poorer nations are generally subjected to the highest burden of cases. In Florida, other than typical factors such as travel, the presence of competent vector populations, and population density, specific factors that may lead to autochthonous transmission of DENV remain unclear and is being actively investigated (Munoz-Jordan *et al*., 2013; Anez *et al*., 2012).

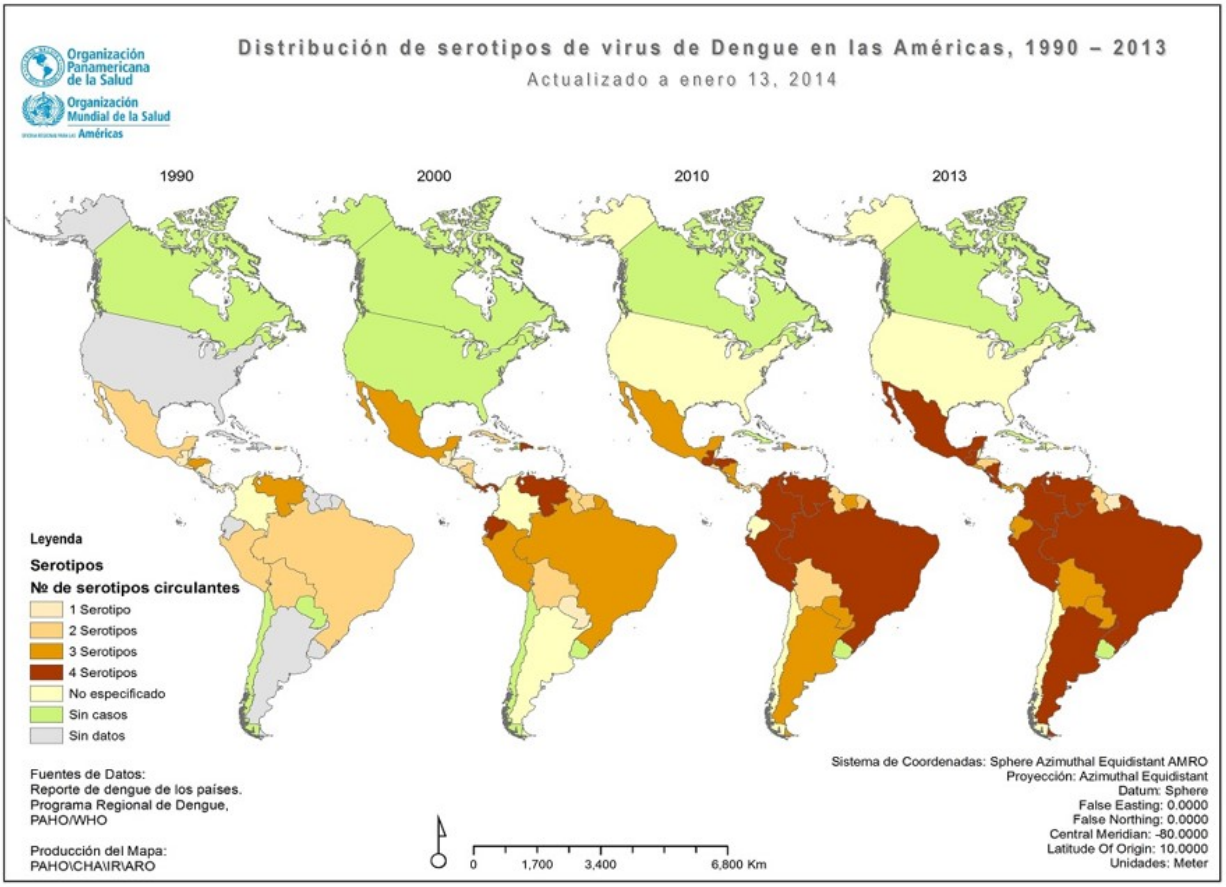

Fig. 1.3: Distribution of DENV serotypes in the Americas, 1990-2013. These maps illustrate the number of DENV serotypes present in the Americas from the time period 1990-2013 using data from four representative years. Note that by 2013 all four serotypes were found to be present in nearly the entire region south of the United States. (PAHO, 2014)

1.1.3.6. DENV - Importance in Florida

After a 75 year absence, local transmission of DENV was documented in Florida, initially during an outbreak of DEN1 (genotype V, clade E) in Key West during 2009-2010. It is now considered as triansiently present within FL. One-hundred and three (103) autochthonous cases were documented in the time period between 2009-2012 with the majority of those cases (27 in 2009 and 63 in 2010) associated with an outbreak in Monroe County (Key West). However, epidemiologically unrelated, locally-acquired cases were also documented in Broward, Hillsborough, Miami-Dade, Palm Beach, Osceola, and Seminole counties through 2012. An additional outbreak of a genotype not derived from the Key West strain occurred in Martin County during 2013. (Florida Health, 2015; Anez and Rios, 2013; Munoz-Jordan *et al*; 2013; Adalja *et al*., 2012; Anez *et al*., 2012) Two introductions of local transmission thus are thought to have occurred in Martin County, the first near Port Salerno in 2011 and the second near Jensen Beach; the latter introduction was responsible for the outbreak (Florida Health, 2015). In 2016, an autochthonous case of DEN4 (Cone, *personal comm*.). Another dengue case was detected in Miami-Dade during the 2016 Zika virus outbreak investigations and was found to be locally-acquired (Florida Health; 2016; Miami Herald, 2016).

Within Florida, the Miami metropolitan area is arguably the area at highest risk for introduction of the virus. However, limited transmission has, in fact, occurred in the area, as some isolates from the area match sequences derived from the 5' end of the genome (C and prM/M respectively) of isolates obtained from the Martin County outbreak (genotype V, clade C) (Munoz-Jordan, *et al*., 2013). Although focusing on specific genome regions may more easily identify genotypic differences, it has been asserted by Christenbury *et al*. (2010) that for epidemiological reasons, a method for rapid full-length genome sequencing be implemented to better elucidate transmission patterns in epidemic situations.

1.1.3.7. DENV - Control Measures

Before 2015 when a licensed dengue vaccine for all four serotypes became available on the market in a number of countries, control of vector populations was the only method available to combat

DENV transmission. The control of dengue was unamenable to both preventive and prophylactic medical interventions. The efficacy and safety of the available vaccine remains to be seen but has shown promise. (Wichmann *et al*., 2017; Petersen and Barrett, 2009) While vector-control measures have proven successful where there is adequate infrastructure in place, this is severely lacking in a large portion of the world. There are issues with many 'blanket approach' vector-control measures that affect the local ecology, sometimes detrimentally (e.g., DDT), and that they must be undertaken indefinitely (Patterson, 2004). Vector control measures include mechanical, chemical, and biological methods. The favored means of each, respectively, include the removal of standing water from potential breeding sites, fogging with the organophosphate pyrethroid, temephos, and treatment with aerial spraying of *Bacillus spp*., notably *Bacillus thuringiensis isrealensis* and *B. sphaericus*, which target mosquito larvae. However, due to limitations of each approach and the benefits associated with using multiple approaches, an integrated approach to vector control is currently favored. (Becker *et al*., 2003) Novel approaches to vector management via biological methods include transgenic modification to reduce mosquito populations and the reduction of vector competency via methods such as the introduction of symbionts or parasites. (Patterson, 2004; Becker *et al*., 2003) For instance, a UK firm, Oxitech, has recently released male-lethal genetically modified (GM) *Ae. aegypti* mosquitoes (OX513A) in the Cayman Islands, Malaysia, Brazil, and Panama, with trials also set to occur in Key West, FL (NPR, 2016; Carvalho *et al*., 2015; Gorman *et al*., 2015; Winskill *et al*., 2015; Harris *et al*., 2012; Lacroix *et al*., 2012; Harris *et al*., 2011). The major questions regarding this approach and other vector modifications is related to fitness and longevity, however dramatic reductions in *Ae. Aegypti* populations have been observed in the short term due to the release of these GM mosquitoes. What will occur over the long term at release sites remains to be seen.

While vector control methods for combatting dengue transmission are time-proven and effective, they cannot universally prevent or eliminate dengue. As with most infectious disease entities, a vaccine remains the 'silver bullet'. Wichmann *et al*., 2017 and Webster *et al*., 2009 provide thorough reviews of the development, progress, and remaining questions regarding dengue vaccines. Briefly, each group details that there are at least 12 different vaccine candidates for dengue virus in various stages of licensure, clinical trials or pre-clinical development. A chimeric, infectious preparation (ChimeriVax) that

includes prM and env proteins of the four DENV (and is thus tetravalent) successfully underwent phase 3 clinical trials and is now licensed for use in a number of countries in Latin America and Asia under the trade name Dengvaxia® (Sanofi Pasteur, Swiftwater, Pennsylvania). Each group also discusses other developments; there are two each of monovalent vaccines prepared from live-attenuated and infectious clones, respectively. Preparations using immunogenic viral proteins, viral DNA, and complete vaccines using either inactivated virus or recombinant technologies (replication incompetent, virus vectors) are also in development. A functional, efficacious DENV virus vaccine would be of enormous value and reduce the burden on, or the need for, all other control measures.

1.1.3.8. DENV - Clinical Disease and Pathogenesis

DENV causes a spectrum of illness in humans from a mild febrile presentation all the way through to fatal outcomes. Infection may also be asymptomatic. A minority of patients may progress to severe illness where the development of hemorrhagic complications and/or shock may occur late. (WHO, 2009) The main type of cell infected by DENV is thought to be dendritic cells (DCs) after inoculation of the host via mosquito (Boonak *et* al., 2010; Sun *et al*., 2009; Boonak *et al*., 2008). Migration of these infected cells to regional lymph nodes is then thought to lead to systemic infection where cells such as hepatocytes and endothelial cells (ECs) may also be infected or play a role in pathogenesis (Azizan *et al*., 2009; Azizan *et al*., 2006; Peyrefitte *et al*., 2006; Dewi *et al*., 2004). In secondary infections, monocytes and macrophages are thought to play an additional source of infection, and play an extremely important role in the onset of severe illness (Wati *et al*., 2007).

Antibody-dependent enhancement (ADE) of infection is the current dogma that most dengue researchers adhere to with respect to the development of severe pathogenesis. The ADE model was based on field observations by Halstead, 1976 referring to the preponderance of Thai patients that manifested severe illness upon secondary, heterotypic infections with dengue. In this theory, non-neutralizing cross-reactive antibodies produced in response to a primary infection with dengue virus are thought to increase the severity of illness in heterotypic, secondary DENV infections. FcγR-bearing cells such as those of the monocyte/macrophage lineage are thought to be the primary cellular targets in ADE

via intracellular viral escape from virus-antibody complexes once endocytosed (Boonak *et* al., 2010; Honda *et al*., 2009; Boonak *et al*., 2008; Chen *et al*., 2008; Fink *et al*., 2006; Huang *et al*., 2006; Kontny *et al*., 1988). Uptake into and subsequent virus replication within these cells is thought to elicit a maladaptive cytokine response, termed the 'cytokine storm', that ultimately produces the vasculopathy that is the hallmark of severe cases (Basu and Chaturvedi, 2008; Fink *et al*., 2006). Provided that normally targeted cells, such as dendritic cells are also still infected, ADE should therefore increase the total viral load, however the opposite seems to be true (de la Cruz-Hernandez *et al*., 2013; Tricou *et al*., 2011). Alternatively, chemotactic cytokines such as monocyte chemoattractant protein 1 (MCP-1), that have been found to be upregulated in severe illness (Lee *et al*., 2006), may also serve to increase the number of infected cells by recruiting newly susceptible monocyte-derived macrophages to infected cells (Sierra *et al*., 2010). The infection of these recruited cells would then be dependent on the co-localization of non-neutralizing antibody. Cells of the liver and the endothelium are also known to play a role in ADE but whether through direct infection or some other means remains to be seen (Avirutnan *et al*., 2007). The exact molecular mediators involved in ADE and the direct roles of other implicated cell-types have been intensively investigated yet not fully elucidated.

Cytotoxic T-cells have also been implicated in severe illness, such as those low-avidity cross-reactive types that are induced in ADE, but again their exact role is unclear. CD8+ T-cell mediated cytotoxicity is coincident with a shift to a T_H1 mediated response. It is thought that secondary infections with DENV exhibit a shift to cell-mediated immunity, likely after the initial memory-induced humoral response, and that this response is damaging. This theory does not require the infection of macrophages or other Fc_γ-receptor cells for the development of severe illness, but may in fact be exacerbated by ADE. A shift to cell-mediated immunity would be unlikely after an appropriate humoral response and would require an active (non-neutralized) infection. (Basu and Chaturvedi, 2008; Dong *et al*., 2007) The expansion of low affinity CD8+ T-cells that are inefficiently cytotoxic in secondary infections would seem to support this idea of a continuous and inappropriate response (Fink *et al*., 2006). This shift to cell-mediated immunity was found to be concurrent with defervescence in 20 Indian dengue patients, thus at a time of decline in viral titers. Numerous pro-inflammatory mediators were found to be produced in these same 20 Indian patients. Furthermore, 10 patients with severe illness had elevated levels of intracellular

macrophage inflammatory protein 1 alpha (MIP-1α) and IFN-γ compared to the 10 non-severe cases, indicating upregulation of these cytokines. (Kadhiravan *et al*., 2010) This should not, however, discount the role that cytotoxic T-cells may play in the development of severe pathology, instead, that their role also seems to be closely tied to secondary infections.

Alternatively, cytotoxic T-cells may contribute to pathogenesis when there is greater viral burden (regardless of mechanism) by inducing cell death and the subsequent release of elevated levels of previously intracellularly sequestered viral proteins. These proteins in turn would be presented in an MHC-I manner to additional CD8+ T-cells that can be activated to produce more soluble mediators further downstream, again in an elevated response. This was evident in a study that found a large proportion of T-cells reactive for DENV NS3 protein in peripheral blood mononuclear cells (PBMC)s of Thai patients with severe dengue. DENV NS3 protein functions as a serine protease, is involved in processing the viral polyprotein, and would almost exclusively be found intracellularly, although this phenomenon would seem to require some amount of surface expression. (Duangchinda *et al*., 2010) The elevated detection of various cytokines that are produced mainly via cytotoxic T-cells both *in vitro* and *in vivo* indicating severe illness also implicates a role for CD8+ T-cells (Basu and Chaturvedi, 2008; Fink *et al*., 2006).

The generation of normally beneficial inflammatory mediators in quantities that become damaging has been found as a mechanism involved with severe dengue pathology. The generation of a 'cytokine storm' is not exclusive from other proposed mechanisms, and in fact would be dependent on the occurrence of at least one mechanism acting upstream. While the sequence of cascades and the exact players involved have yet to be fully elucidated, TNF-α and IFN-γ seem to be two of the most important mediators and are often elevated in severe illness. (Basu and Chaturvedi, 2008; Fink *et al*., 2006) ILs-1β, 2, 4, 6, 8, 10, 12, macrophage migration inhibitory factor, granulocyte-macrophage colony stimulating factor (GM-CSF), MCP-1 or CCL2, elastase, sTNFR, regulated and activated upon normal transcription expressed and secreted (RANTES), MIP-1α, MIP-1β, sIL-2R, IL-1ra, interferon-γ inducible protein (IP)-10 or CXCL10, soluble intercellular adhesion molecule (sICAM-1), soluble vascular cell adhesion molecule (sVCAM)-1, and vascular endothelial growth factor (VEGF) have all been implicated to play a role in severe illness and more appropriately illustrate the 'storm'. (Appanna *et al*., 2014; Rathakrishnan *et al*., 2014; deOliveira-Pinto, *et al*., 2012; Gupta *et al*;, 2012; Puerta-Guardo *et al*., 2012; Thayan *et al*., 2010;

Azizan *et al*., 2009; Sun *et al*., 2009; Basu and Chaturvedi, 2008; Boonnak *et al*., 2008; Chen *et al*., 2008; Restrepo *et al*., 2008; Yen *et al*., 2008; Azizan *et al*., 2006; Halstead, 2007; Dong *et al*., 2007; NE Reis *et al*., 2007; Azizan *et al*., 2006; Fink *et al*., 2006; Shmitt *et al*., 2006 Shresta *et al*., 2006; Lin *et al*., 2005; Mangada and Rothman, 2005; Avila-Aguero *et al*., 2004; Dewi *et al*., 2004; Bosch *et al*., 2002; Suharti *et al*., 2002).

In a Malaysian study (Appanna *et al*., 2012), cytokine profiles were measured for 27 different markers in 13 classical dengue patients and 29 DHF patients against 6 healthy individuals. These profiles were measured at different time intervals, including 2-3, 4-6, and more than 7 days after onset for patients. One curious result was that IFN-γ was seen to be elevated in DHF patients versus non-severe cases for the duration of illness. The production of IFN-γ in these non-severe cases was also lower than that for healthy controls. This study also showed that IP-10, RANTES, MCP-1, MIP-1β, and ICAM-1 were generally more pronounced in DHF patients, followed by non-severe cases, and then healthy controls; these responses were individually time-dependent. Furthermore, DHF patient sera was found, by confocal microscopy, to be responsible for altering the tight junctions of vascular endothelial cells more profoundly than non-severe cases and healthy individuals. The fact that vascular permeability goes hand in hand with the development of shock shows that this particular result supports current theories of severe DENV pathogenesis. There was no mention in this study of specific infecting serotype nor direct mention of previous infection status.

A study in the Philippines, by Chagan-Yasutan *et al*., 2013, took a similar approach with respect to measuring cytokine and chemokine levels in DENV infected patients but also chose to focus on levels produced during the 'critical phase' to assess predictive values for progression to severe disease. Here they assert that the increased levels of Galectin-9, IFN-α2, eotaxin, and MCP-1 were capable of predicting 92% of DHF cases (out of 29 potential markers) although when averaged among all DENV patients there were a number of markers that were more elevated or equally elevated as these four. A Colombian group, in Restrepo *et al*., 2008, further asserts that these responses to infection with DENV, at least a proportion of them, may be related to ethnicity. Colombian Mestizos showed an altered response to infection than Afro-Colombians with respect to production of TNF-α and IL-6. On the other hand, IFN-γ production was only seen to be elevated in DHF cases over non-severe cases, regardless of ethnicity. It

is unclear, what, if any, confounding factors exist between these populations aside from ethnic background.

It cannot be overstated that it is desirable that a form of 'reference values' exist for these markers in determining potential outcomes of DENV illness. One of the biggest questions regarding the manifestation of shock is the degree of involvement of endothelial cells (ECs) and may be answered by analyzing cytokine responses (Appanna *et al*., 2012; Dalyrymple and Mackow, 2012; Azizan *et al*., 2009; Yen *et al*., 2008; Azizan *et al*., 2006; Lin *et al*., 2005; Dewi *et al*., 2004; Huang *et al*., 2000). If endothelial cells are productively infected and cell death occurs, or if they are damaged in some indirect way, then plasma leakage is a direct result of damage to the vasculature rather than actions of the ECs themselves. Endothelial cells have been infected with DENV *in vitro*, but the occurrence of this *in vivo* is questionable. Uptake of virus-antibody complexes by ECs has been noted to induce cytotoxic mediators (Fink *et al*, 2006). Indirect (bystander) damage to the vasculature has been implicated by numerous inflammatory mediators such as reactive nitrogen and oxygen species, TNF-α, IFN-γ, and as discussed below, complement (Basu and Chaturvedi, 2008; Fink *et al*., 2006). Importantly, self-treatment with over-the-counter drugs such as aspirin may also contribute to complications such as hemorrhage (Valerio *et al*., 2006). Other serum markers indicative of angiogenesis may be directly linked to repairing damage to the vasculature.

Upregulation of vascular endothelial growth factor (VEGF) in DENV infected HPMEC endothelial cells has been demonstrated *in vitro* and has been found to be elevated in human sera (Azizan *et al*., *unpublished data*; Azizan *et al*., 2009; Azizan *et al*., 2006). However, the detection of VEGF as a serum marker in a significant quantity of patients that have developed severe illness would be desirable. It has also been noted that studies attempting these analyses may be hampered by extravasation of serum markers if sampling during shock and thus difficult to accurately measure (Kadhiravan *et al*; 2010). The way in which shock may arise is due to induction by the host, perhaps in direct response to the damaging 'cytokine storm'. This would indicate the involvement of endothelial cells during DENV infection being limited to the temporary relaxation of the tight junctions responsible for maintaining vascular integrity through mediators such as histamine and prostaglandins. Both phenomena play a role in the

development of plasma leakage and shock in severe dengue illness (Fink *et al*., 2006). Finally, it is noted by most, that the limitations of these individual studies are small sample sizes.

Complement in the form of the membrane attack complex (MAC) as well as anaphylatoxin (C5a) may also play a role in the development of severe illness (Shresta, 2012). DENV NS1 may be responsible for this association as levels of both complement products were shown to be positively correlated with levels of NS1 in 9 Thai patients with shock (Avirutnan *et al*., 2006). C5a has both a direct and indirect effect on increasing vascular permeability (Moore, Jr., 2004). Complement, NS1, antibody generated to NS1, and platelets also appear to interact in a manner that promotes severe illness. Anti-NS1 antibodies apparently cross-react with platelets and subsequently induce lysis via complement leading to coagulopathy. This coagulopathy, if sufficient, promotes the development of hemorrhage and/or plasma leakage. These cross-reactive antibodies are of the IgM class indicating an acute, often primary infection, as IgM is often undetectable in secondary cases. (Fink *et al*., 2006) Perhaps this interaction is largely dependent on virus burden only, as suggested by the association with serum levels of NS1 and the acute chronology of the phenomenon.

ADE has been documented *in vitro* but has been difficult to assess in humans (Midgley *et al*., 2011; Halstead, 2007; Halstead *et al*., 1983; Halstead and O'Rourke, 1977). Support for this theory further comes from epidemiological data demonstrating increases in severe illness following sequential introductions of heterotypic dengue serotypes in naïve populations. This was seen in Cuban epidemics after introduction of DEN2 (1982) and DEN3 (1997) following the original introduction of DEN1 (1977) (Guzman *et al*., 2010; Halstead, 2007). It is currently unknown if ADE extends to other non-DENV members of the *Flaviviridae*. It has not been documented in JEV or YFV vaccinees subsequently infected with DENV, yet it may or may not occur in both. ADE may have indeed occurred in a natural WNV infection whereby a Florida man with pre-existing antibody to dengue succumbed to a fatal, and most unusual, hemorrhagic WNV infection (Paddock *et al*., 2006). WNV is not known to cause this type of pathology in the Western hemisphere.

Another theory attempting to describe the manifestation of severe DENV illness, is that based on viral load. The theory that increased viral load mediates severe illness holds that the higher the titer of virus that is generated, the greater the chance of developing severe disease (Gubler *et al*., 1981).

Increased viral burden may arise in numerous ways. For instance, increased viral load may be a consequence of ADE. However, it has been noted in studies that secondary cases result in both decreased peak viremia as well as lower levels of DENV NS1 antigenemia (Tricou *et al*., 2011). Increased viral burden is also dependent on the infecting dose and may be affected in one of two ways. A mosquito may itself have a greater viral load, or a person may become 'hyper-infected' from multiple mosquitoes. An increase in viral load may also be conferred by either, or both, host and virus genetics in some as yet undetermined way (Guzman *et al*., 2010). A total increase in viral load may have one of two effects: the immune response may be greatly amplified, becoming maladaptive and damaging, or viral progeny may overwhelm the immune system, directly causing an increased amount of cell death. Elevation of cell-mediated cytotoxicity, in addition to a 'cytokine storm' may both result from increased viral burden. Evidence for this mechanism at work in dengue patients in a Taiwanese study showed that increased viral burden resulted in both a delay of viral clearance and increased detection of virus-antibody complexes in 49 DEN2 patients suffering from severe illness compared to 54 cases of uncomplicated forms; all of these patients were determined to have secondary infections (Wang *et al*., 2006). C5a and the membrane attack complex (MAC), also discussed above, were found to be elevated in severe cases. This can be taken in conjunction with reports that elevated levels of DENV NS1 protein (a surrogate marker for increased viral burden) positively correlate with the development of severe illness. (Avirutnan *et al*., 2006; Libraty *et al*., 2002).

Host genetics likely plays a part in severe cases but the current paucity of data does little to enhance our understanding of what factors may predispose an individual to developing severe illness. Human leukocyte antigen (HLA) type has been implicated by observations that people of African descent appear to have some measure of protection from developing severe manifestations of dengue and a number of polymorphisms seem to partially confirm this (Guzman *et al*., 2010; Fink *et al*., 2006). A single nucleotide polymorphism (SNP), AA to AG, in the gene encoding for DC-SIGN (a putative receptor for DENV) may in fact predispose an individual to severe illness. Dendritic cells derived from 20 Taiwanese volunteers bearing the AG genotype showed increased DC-SIGN expression, TNF-α, IL-12p40, and IP-10 production, yet decreased viral replication compared to those derived from the AA SNP. In the same

study, analysis of a Taiwanese cohort of dengue patients also showed significant association of severe illness with the polymorphisms AG or GG. (Wang *et al*., 2011)

As there is a mechanistic threshold separating uncomplicated dengue from severe illness, there should also be one separating DHF and DSS. This could simply be attributed to additionally elevated viral loads and/or cytokine responses (Appanna *et al*., 2012; Rathakrishnan *et al*., 2012; Dalyrymple and Mackow, 2012; Azizan *et al*., 2009; Sierra *et al*., 2010; Yen *et al*., 2008; Azizan *et al*., 2006; Lin *et al*., 2005; Dewi *et al*., 2004; Suharti *et al*., 2002; Huang *et al*., 2000). Alternatively, it may be that DSS is predominantly mediated through some undetermined host genotype, such as inconclusive investigations of polymorphisms within the TNF-α gene (Guzman, *et al*., 2010). However, there is no consensus model for describing the precise cascade of events leading to the development of shock in severe cases of dengue. This may be because, from what can be assessed from the literature, that there is no single model that describes the phenomenon as it occurs *in vivo*. Nevertheless, one of the major players in producing severe illness is tumor necrosis factor alpha (TNF-α). Synergistic effects of TNF-α with cytokines such as interferon (IFN)-γ and interleukin (IL)-8 have been under investigation in the development of DENV illness. (Sierra *et al*., 2012; Restrepo *et al*., 2008; Yen *et al*., 2008; Talavera, *et al*., 2004)

TNF-α is a potent mediator of inflammation and possesses a variety of signaling capabilities. TNF-α is also considered a pyrogen in that it induces fever and has anti-angiogenic properties. These properties serve to make it an important factor in dengue associated shock. It is a basic protein that associates into 3 subunits existing in two forms; the first is a 27 kD membrane bound form. This membrane-anchored protein, mTNF-α, may then undergo proteolytic cleavage by the metalloprotease, TNF-α converting enzyme (TACE), to form the soluble, 17 kD form, sTNF-α. The membrane bound form primarily acts in an autocrine/paracrine manner, while the secreted form functions in an endocrine capacity. (Parameswaran and Patial, 2010) The most significant producers of TNF-α include monocytes and macrophages. These cells are also thought to be important mediators in the development of severe illness with dengue virus (DENV) infection. (Vinh and Holland, 2009) Two different receptors that bind to TNF-α, TNFR1 and TNFR2, are expressed on different cell populations. TNFR1 is expressed rather ubiquitously on most cell types, while TNFR2 is limited to expression on cells of lymphoid origin.

Importantly, TNFR2 is fully activated only in the presence of mTNF-α and not sTNF-α, necessitating direct cell-cell interactions. Chemotactic molecules produced in response to DENV infection can thus have an impact of the likelihood and degree of TNFR2 activation. Upon binding of its cognate receptors, TNF-α initiates a number of distinct intracellular processes via cascade events. The downstream effects of gene regulation through TNF-α signaling are multifarious, but can be broken down into two ultimate effects, one promoting apoptosis and the other cell survival. Apoptosis is mediated through binding of TNFR1 and the production of caspase8. Conversely, binding of TNF-α to either receptor may lead to anti-apoptotic effects, proliferation of immune effector cells, and subsequent production of inflammatory cytokines. (Parameswaran and Patial, 2010)

Monocytes/macrophages are thought to be both the major producers of TNF-α and the major target cells for dengue virus in severe illness. Therefore, it is reasonable to conclude that the response of these cells to infection may lead directly to an increase in vascular permeability and development of shock under certain conditions (Fig. 1.4). Most studies implicate a major role for TNF-α and, subsequently, IL-8, as levels of these cytokines have been found to be elevated in DENV infections and extremely elevated in severe illness (or models of severe illness). One consequence of the activation of nuclear factor (NF)-κB by TNF-α includes the downstream production of the cytokines IL-6 and IL-8. NF-κB promotes inflammation and a general anti-apoptotic state and this is mediated in large part via the production of TNF-α. The increase in vascular permeability has been also found to be positively correlated with a number of markers indicative of extravasation of these phagocytic cells such as MCP-1 and ICAMs. (Basu and Chaturvedi, 2008; Lee *et al*., 2006). As previously mentioned, IFN-γ production has been found to be upregulated in severe illness but other authors have found the converse to be true as well (Basu and Chaturvedi, 2008). The former would seem to make more sense as IFN-γ has been found to act synergistically with TNF-α to increase vascular permeability (Pang *et al*., 2007). Elevated concentrations of TNF-α and/or IL-8 have been demonstrated in DENV patient serum, murine models, and various *in vitro* models including endothelial cells, epithelial cells, monocytes, macrophages, dendritic cells, and T-cells (either in direct response to infection or through interactions with infected cells) (Sun *et al*., 2009; Boonnak *et al*., 2008; Chen *et al*., 2008; Restrepo *et al*., 2008; Yen *et al*., 2008; Dong *et al*., 2007; Azizan *et al*., 2006; Shresta *et al*., 2006; Dewi *et al*., 2004; Bosch *et al*., 2002). Additional models

have shown the importance of TNF-α through use of treatments such as dexamethasone, ribavirin, and leaf extracts from *Hippophae rhamnoides*. Treatment with these compounds resulted in increased cell viability and/or decreased viral load with subsequent lower levels of TNF-α production (Jain *et al*., 2008; NI Reis *et al*., 2007).

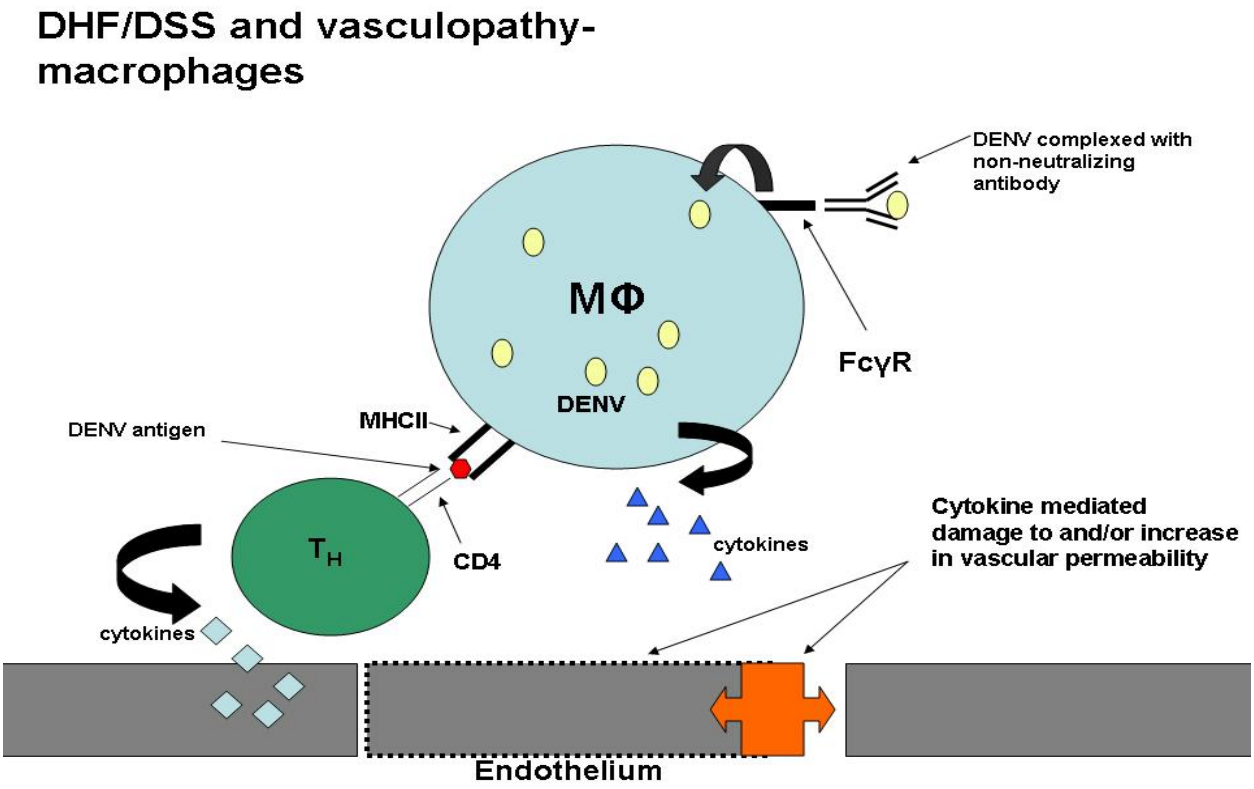

Fig. 1.4: An immunological model for the pathogenesis of severe dengue (DHF/DSS) and coincident vasculopathy. It is thought that DENV infected macrophages (Mϕ) play a dominant role in the ultimate vasculopathy witnessed in severe manifestations of dengue due to their pluripotent roles as immune-effector cells. This includes but is not limited to: their ability to uptake incompletely neutralized virus in a heterotypic, secondary DENV infection which leads to infection of the Mϕ and the resultant cytokine response; as well as their role as antigen presenters which aslo result in cytokine production, here due to the recruitment of T-cells. These cytokines further act upon the vascular endothelium resulting in damage and/or an increase in permeability due to relaxtion of gap junctions. This ultimately leads to plasma leakage and shock. (*Adapted from* Fink *et al*., 2006.)

1.1.3.9. DENV - Diagnosis and Prognosis of Infection

Heart rate (bradycardia), positive tourniquet tests, demonstration of pleural effusion (via X-ray or sonogram), liver enzyme levels such as elevated serum transaminase, altered hematocrit levels, as well as hemoglobin, white blood cell, and platelet counts are all used in determining pathogenesis in DENV

infected patients (Fig. 1.5) (WHO, 2009). In a study of 100 serologically dengue positive patients, these methods were found to be capable of predicting dengue in 81% of patients without the use of traditional confirmatory methods of diagnosis. However, only elevated liver enzymes, specifically, aspartate aminotransferase (AST) was seen in more than seventy percent of patients (73%). Individually, these tests appear less predictive as a smaller proportion of patients exhibited one 'hallmark' or another, in descending order: pleural effusion (63%), leukopenia (57%), elevated alanine aminotransferase (ALT, 43%), positive tourniquet test (40%), hematocrit levels >45% (32%). Another marker but one less acknowledged in the literature, gall bladder wall edema, was observed in 87% of patients and increased the overall predictive value of these non-confirmatory tests. (Manimala Rao, 2014) On the other hand, the fact that this study did not focus on different outcomes of DENV disease in their predictions, *i.e.* non-severe versus severe outcomes, seems to unfortunately devalue some of the collective tests prognostic utilities. For instance, a progressive increase in hematocrit levels concomitant with progressive thrombocytopenia is considered as a 'warning sign' of severe dengue according to WHO guidelines. (WHO, 2009)

Patient IgG and/or IgM ELISAs are still the most widely used and preferred method for confirming diagnosis with dengue. However, only 50% of patients will display detectable levels of IgM, indicative of an acute, usually primary infection, by days 3-5 after onset. IgG only becomes detectable 1 week after illness onset and requires two samples for confirmation (acute and convalescent). In secondary cases, where patients are more likely to develop severe illness, IgG is detectable at onset but only reaches peak titers 7-10 days after onset of illness. Acute and convalescent samples are still required for confirmation (Fig. 1.6). IgM is usually barely detectable during the acute phase of secondary cases and titers do not normally increase significantly. A number of traditional, end-point and real-time RT-PCR assays have been developed for detection of dengue virus RNA in patient sera, now including one real-time assay approved by the FDA for use in US laboratories. RT-PCR has been useful for confirming acute dengue infection as peak viremia, and thus levels of viral RNA, occurs prior to development of antibodies (Fig. 1.7), importantly DENV NS1 levels show a similar profile and can be detected by ELISA (Fig. 1.8). RT-PCR requires more expensive equipment and much more stringent handling of samples and thus is not as widely used as ELISA. It is also of little prognostic value on its own, as higher levels of viremia do not

always correlate with greater disease severity. However, it is confirmatory and is capable of informing infecting serotype. (WHO, 2009)

Obtaining and transporting appropriate samples has also been an obstacle in DENV diagnostics. Whole blood, serum, and/or plasma samples have been the norm for all of the above assays. Cerebrospinal fluid (CSF) has not traditionally been sought to diagnose DENV but testing has been shown to be useful after ruling-out other arboviral illnesses, especially since dengue has been shown to cause neurological illness in rare cases (Goswami *et al*., 2012; Gulati and Maheshwari, 2007). IgM and DENV NS1 have been detected by ELISA in the CSF of fatal cases of infected individuals, though not all involved in the study (Araujo *et al*., 2011). Proper collection and handling of these samples, e. g., maintaining cold-chain procedures and obtaining follow-up specimens, is sometimes difficult and therefore other types of samples have been investigated for use in DENV diagnostics. Dried blood samples collected on filter paper has shown promise in detecting anti-DENV IgM and IgG as well as NS1 antigen. Oral swabs have shown less promise but may be useful in detecting anti-DENV IgA, although interpretation of results may be difficult (Anders *et al*., 2012; Nawa *et al*., 2005). Results, such as those reported in Anders *et al*., 2012, assert that DENV-specific IgA detection is superior in serum and bloodspots rather than oral swabs.

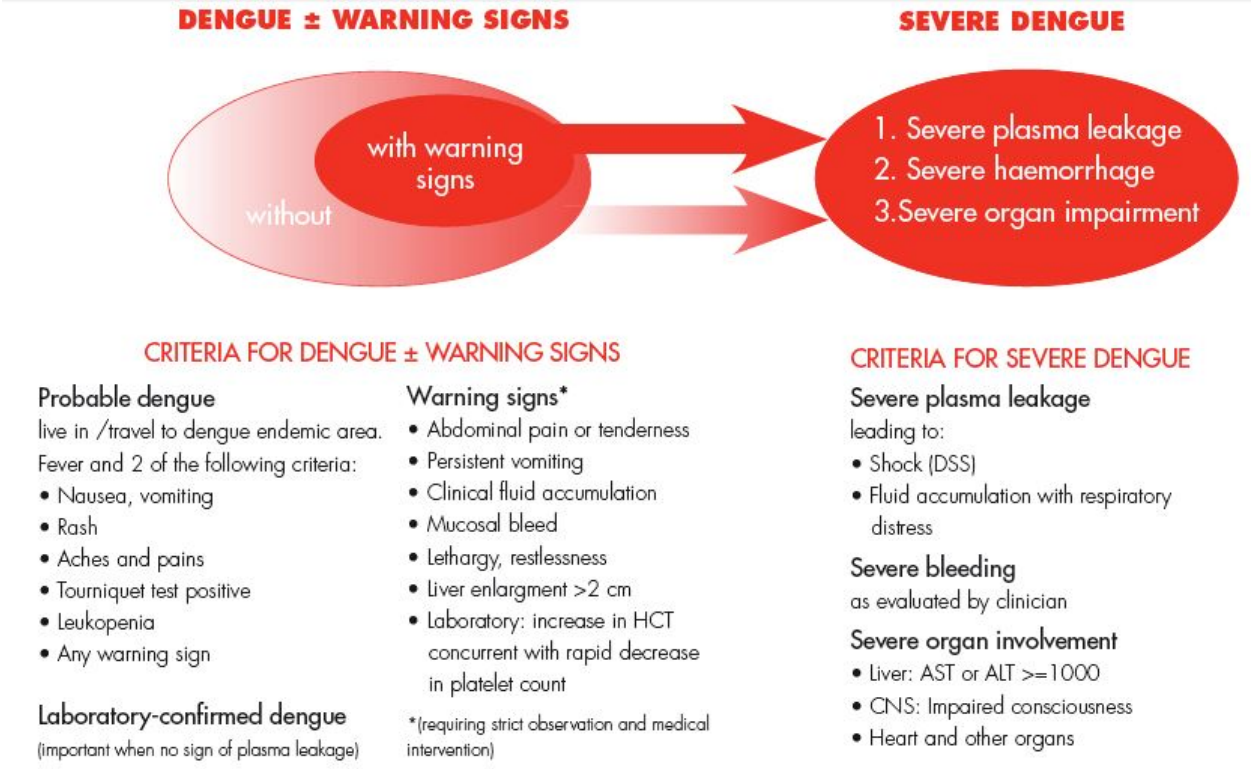

Fig. 1.5: WHO criteria for establishing patients with dengue exhibiting warning signs and those experiencing severe dengue. This figure illustrates four levels of dengue patient diagnosis and prognosis from: 1. probable dengue 2. laboratory confirmed dengue (both for diagnosis) 3. dengue with warning signs and 4. severe dengue (both for prognosis). (WHO, 2009)

Highly suggestive	Confirmed
One of the following: 1. IgM + in a single serum sample 2. IgG + in a single serum sample with a HI titre of 1280 or greater	One of the following: 1. PCR + 2. Virus culture + 3. IgM seroconversion in paired sera 4. IgG seroconversion in paired sera or fourfold IgG titer increase in paired sera

Fig. 1.6: Suggestive versus confirmatory laboratory results for determining infection with DENV. Confirmation of DENV infection can only be achieved in the laboratory by one of four routine methods: a positive PCR result, isolation of virus and evidence of seroconversion in paired sera via IgM or IgG (a fourfold increase in IgG titer is also sufficient). Positive IgM and/or IgG results in single serum samples are considered suggestive only. (WHO, 2009)

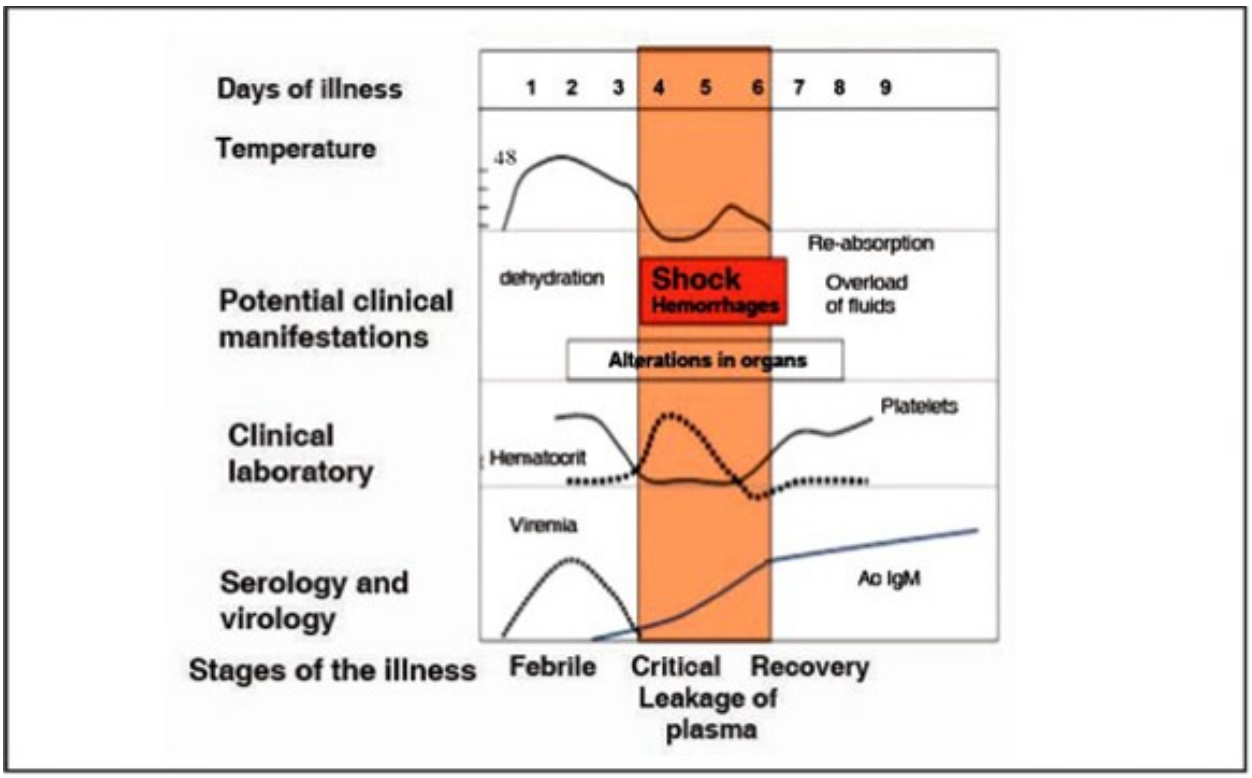

Fig. 1.7: Clinical profile of DENV infection by several parameters. This diagram shows the clinical profile of the different markers and outcomes of infection with DENV according to the following parameters by days post onset: fever (temperature), hematocrit levels, platelet counts, viremia, and anti-DENV IgM levels. It is a desirable goal that patients that would proceed to into shock (shaded area, days 4-6) be identified by some method at a time around peak viremia and fever (days 1-3) in order to ensure proper clinical management. (WHO, 2009)

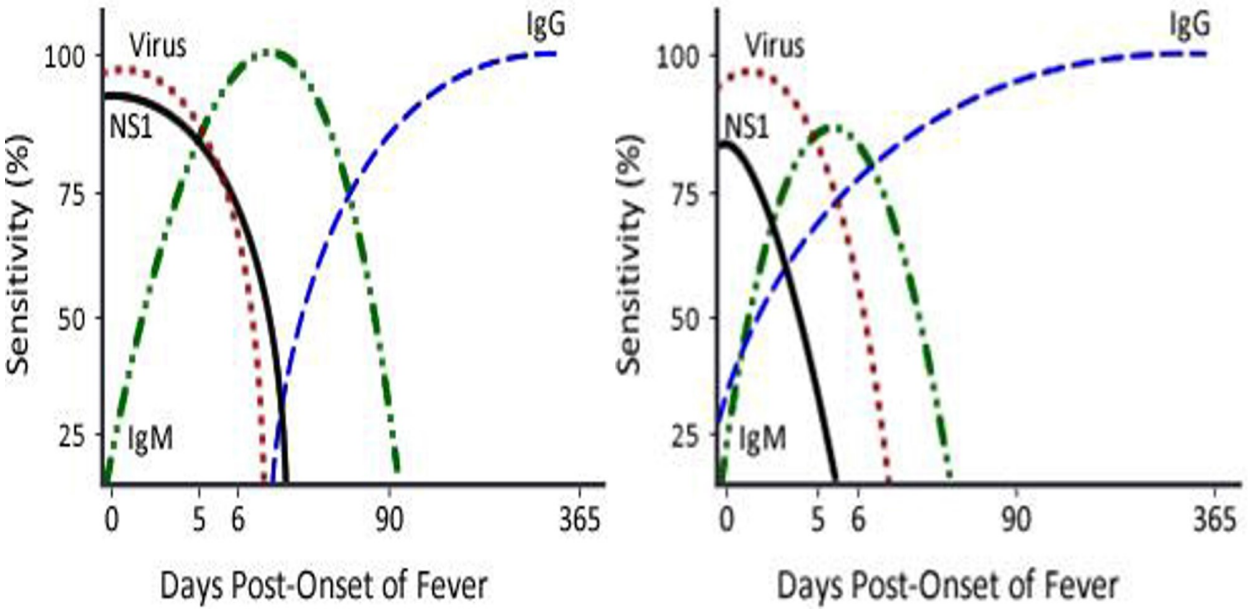

Fig. 1.8: Clinical profile of DENV infection by several parameters. This diagram shows the clinical profile of the different markers of infection with DENV (via sensitivity of assay) according to the following parameters by days post onset of fever: viral detection, NS1 detection, IgM detection, and IgG detection. The left image illustrates profiles for primary DENV infections and the right, non-primary infections. Note that NS1 detections compares favorably to detection of virus in acute cases (especially those that are primary infections). (CDC, 2015)

1.1.3.9.1. Traditional Methods of DENV Diagnosis

1.1.3.9.1.1. Serological Methods of DENV Diagnosis

Serological assays have been the mainstay of DENV diagnostics since their inception and have traditionally been the means used by the FLDOH BPHL-Tampa laboratory (Stark, *personal comm.*). The standard assay has been detection of antibody to DENV via enzyme-linked immunosorbent assay (ELISA). This assay is useful for detecting acute primary and secondary cases as well as past exposure based on the resultant serological profiles. Detection of IgM in acute cases is one of the more useful approaches, especially in primary infections. IgG profiles may also be determined through ELISA but require acute and convalescent sera, most often leading to confirmation after resolution. IgG positive individuals may also indicate previous infection based on profile. However, a marked elevation of IgG

occurs in secondary cases during the acute phase due to the induction of non-neutralizing antibodies generated against heterologous serotypes from previous infections. (WHO, 2009) Due to the (hyper) endemic status of DENV in regions where it circulates it has been difficult to ascertain the longevity of circulating antibody. It is thought to be long lasting though, as it has been reported that four individuals retained circulating antibody to DEN1 after more than 60 years of exposure centered on the outbreak of 1944-1945 in Hawai'i (Imrie *et al*., 2007).

The hemagglutination inhibition assay (HAI) is also suitable for diagnosing DENV infections. This assay traditionally consists of an antigen (usually produced in suckling mouse brain), antibody from patient samples, and goose erythrocytes. These erythrocytes will agglutinate in the presence of certain antigens, including those of DENV. The formation of antigen-antibody complexes prior to treatment with the red blood cells leads to the inhibition of agglutination. Therefore, acute infections are often evidenced by the lack of agglutination after multiple dilutions of the patient sample indicating high circulating titers of antibody. This test is less specific then ELISA as there is significant cross reactivity among the flaviviruses. (Petersen and Barrett, 2009; Perera *et al*., 2008; Seligman, 2008; Calisher *et al*., 1989)

The serum-neutralization plaque-reduction (SNPR) assay, also called the plaque-reduction neutralization test (PRNT) is a confirmatory assay of DENV infection. The WHO provides a guide for this assay in an attempt to reconcile procedural variations while issuing forth minimum requirements (WHO, 2007). In this assay cell cultures are first inoculated with a standardized titer of virus previously incubated with varying dilutions of human sera potentially containing neutralizing antibody. These cultures are then overlaid with a semi-solid agarose-based medium and further treated with a dye that demarcates areas of infected cell death (plaques). Sera lacking specific antiviral antibody show no reduction in the number of plaques generated, while antibody positive samples show a reduction in the number of plaques indicating the presence of virus neutralizing antibodies. This test is very technically demanding, time-to-result may be two weeks for DENV, and results can be impacted by infection status of the individual (primary versus secondary and beyond). There may also be cross-reaction with antibodies within the DENV group as well as other flaviviruses. Finally, even when performed correctly, the results of the SNPR between laboratories may vary due to a lack of standardization, as protocols tend to be generated and modified in-house. (Thomas *et al*., 2009)

Flaviviruses exhibit a great deal of cross-reactivity in traditional serological assays (Petersen and Barrett, 2009; Perera *et al*., 2008; Seligman, 2008; Calisher *et al*. 1989) hampering diagnostic efforts where multiple viruses co-circulate. While serotyping dengue virus isolates may not be a priority when using serological assays such as ELISA for diagnosis, ruling out neurotropic flaviviruses such as JEV, SLEV, and WNV would be desirable. The explosive appearance of the potentially cross-reactive ZIKV has only served to reinforce this point (Landry and St. George, 2017). There are two ways to describe this cross-reactivity; the first is due to actual genetic conservation among the group and subsequent expression in viral proteins. This conservation ultimately leads to the generation of truly cross-reactive antibodies in humans upon infection. On the other hand, highly virus species-specific antibodies are also generated during human infections and these are the most effective at neutralizing virus.

The second way to describe serological cross-reactivity is as an artifact of the assays used to detect flaviviruses. To abrogate this obstacle in diagnostics, an approach that seems to have been borrowed from the design of primers for use in polymerase-chain reaction has been recently employed. Advances in the field have led to the development of novel peptides that are virus species-specific and can either detect virus directly or the antibodies generated against them. Thus, cross-reactivity is evidenced in serological analysis of flaviviruses due to antibodies that are generated against highly conserved epitopes. Yet, cross-reactivity is not a necessary component of diagnostic assays; rather it is a by-product of using complete antigens and/or polyclonal, cross-reactive antibodies rather than species-specific antigens and/or antibodies (i.e. monoclonal). This fact has been addressed and exploited in newer ELISAs such as those directed at both DENV env and NS1, where serotyping has also become possible thanks to the creation of serotype-specific monoclonal antibodies. (Ding *et al*, 2011; Midgley *et al*, 2011; Castro-Jorge *et al*., 2010) Other methods of laboratory diagnosis, such as virus isolation and traditional antigen detection, have even greater drawbacks such as length to result and undesirable sampling requirements (Figs. 1.9 and 1.10).

1.1.3.9.1.2. Histological Methods of DENV Diagnosis

Histological preparations may be used under the greater umbrella of DENV diagnostics such as where material is obtained at autopsy from fatal cases. Histological changes have and will continue to contribute to gaining a greater understanding of DENV pathogenesis but requires special circumstances and a high degree of expertise. These samples are also amenable to immunohistochemical techniques where the targeted detection of host and viral markers may be obtained, for instance, by fluorescent staining. However, given the conditions required above in addition to the other diagnostic assays available, collectively these methods are rarely used. (Petersen and Barrett, 2009)

1.1.3.9.1.3. Virus Isolation of DENV via Culture

Culturing DENV from patient samples is considered the ultimate gold standard in diagnostics as with all infectious etiologies. This requires viral replication in susceptible cells whether they be primary, such as PBMCs, or continuous cell lines, such as VERO (African green monkey, *Cercopithecus aethiops* – kidney, epithelial) and C636 (mosquito, *Aedes albopictus*-larva) lines. Cytopathic effect (CPE) is often, but may not always be evident in culture systems, however, indirect downstream methods are often used to confirm infection. These methods to detect the presence of DENV are multifarious and include virus plaque assay, detection of the viral genome by RT-PCR (now often via fluorescent signals in real-time RT-PCR), and the detection of viral proteins by immunofluorescent assays. Production of disease in an *in vivo* model after recovery of virus establishes a direct etiological relationship. A diagnostic assay then may either measure the presence of virus directly (genome or protein detection) or indirectly (a marker of host response such as specific immunoglobulin production against the virus). Finally, within these assays these markers may be either measured directly (CPE, electron microscopy) or indirectly (colormetric or fluorescent changes). Growth of DENV in cultures may take longer than one week post inoculation. Depending on the type of assay used for confirmation these tests only serve to add to the time until diagnosis. Maintaining the viability of virus in collected samples is also more difficult to produce than preserving viral RNA and proteins or host antibody. For these reasons, virus isolation is used far less

than other diagnostic assays. Virus isolation may be of use where it is desired to obtain virus stocks of circulating strains and for additional genetic and epidemiological studies. Isolates from these cultures may then be subjected to downstream assays such as genomic sequencing. (Munoz-Jordan *et al*., 2013; Leparc-Goffart *et al*., 2009; Petersen and Barrett, 2009)

1.1.3.9.1.4. Molecular Methods of DENV Diagnosis

The detection of DENV RNA in patient samples gained traction in the early 1990s and one of these methods, a nested version of RT-PCR, may be used to provide cDNA for downstream partial genome sequencing. This particular assay is capable of detecting DENV as group during the initial amplification and further amplification allows serotyping of the isolate (Lanciotti *et al*., 1992). The utility of real-time quantitative RT-PCR has led to a number of methods appearing within the literature. One of these methods has been recently approved for diagnostic use in the US by the FDA. This method is serotype-specific and is available either in a single-well, multiplex format or in a single analyte, multiple well format (CDC, 2015).

The usefulness of RT-PCR in DENV diagnostics has enabled our diagnostic capabilities to move even closer to disease onset and detect infection in more acute cases. These assays have also allowed the laboratory to gain an easy way to serotype DENV in patient samples. While the use of RT-PCR has proven superior in many aspects with regards to DENV diagnostics, it has historically been both prohibitively expensive and prone to process issues. Regarding expense, samples first require isolation of nucleic acids adding to both labor and reagent costs. Second, the progressive advancements made from end-point to real-time qRT-PCR have, while reducing time-to-result, added greatly to cost in both platform (hardware and software) as well as reagents. RT-PCR assays, especially real-time platforms, have proven prone to contamination, as these assays are dependent on the exponential amplification of a target, in addition to other process related concerns such as inhibitory factors and sample degradation. Genomic sequencing of isolates, whether partial or full-length, has also proven useful and enlightening but play more of a role in epidemiology than diagnostics. (Munoz-Jordan *et al*., 2013; Leparc-Goffart *et al*., 2009; WHO, 2009; Petersen and Barrett, 2009; Altshuler, 2006)

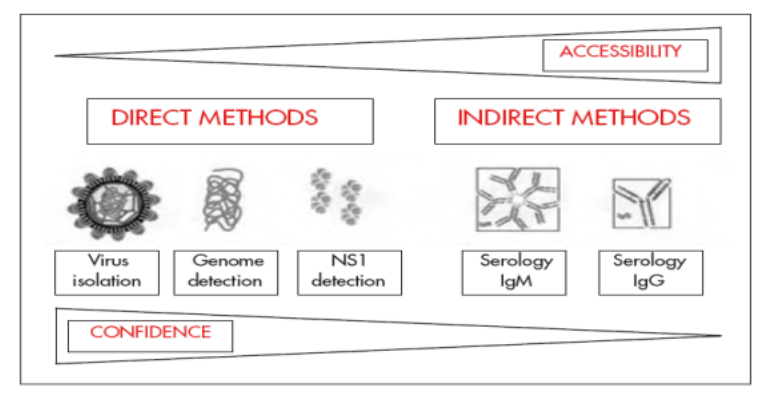

Fig. 1.9: Direct and indirect methods for laboratory confirmation of DENV infection. The direct methods for laboratory confirmation of dengue include detection of the virus either in whole (virus isolation) or in part (genomic detection, NS1 detection) while indirect methods include serological assays detectiong antibody to DENV (IgM, IgG). The direct methods of detection are more reliable but less accessible and indirect methods, vice versa. (WHO, 2009)

Diagnostic methods	Diagnosis of acute infection	Time to results	Specimen	Time of collection after onset of symptoms	Facilities	Cost
Viral isolation and serotype identification	Confirmed	1–2 weeks	Whole blood, serum, tissues	1–5 days	Mosquito or cell culture facilities, BSL-2/BSL-3[a] laboratory, fluorescence microscope or molecular biology equipment	$$$
Nucleic acid detection	Confirmed	1 or 2 days	Tissues, whole blood, serum, plasma	1–5 days	BSL-2 laboratory, equipment for molecular biology	$$$
Antigen detection	Not yet determined Confirmed	1 day >1 day	Serum Tissue for immuno-chemistry	1–6 days NA	ELISA facilities Facilities for histology	$$ $$$
IgM ELISA IgM rapid test	Probable	1–2 days 30 minutes	Serum, plasma, whole blood	After 5 days	ELISA facilities No additional supplies	$
IgG (paired sera) by ELISA, HI or neutralization test	Confirmed	7 days or more	Serum, plasma, whole blood	Acute sera, 1–5 days; convalescent after 15 days	ELISA facilities BSL-2 laboratory for neutralization assay	$

[a] Requirements may vary according to each country's national policies.

Fig. 1.10: Summary of operating characteristics and comparative costs of dengue diagnostic methods. Thisfigure illustrates the methods available to the clinical laboratory for use in DENV diagnostics. Here, the pros an cons of each method are listed including (from left to right): whether an assay is considered confirmatory for diagnosis of DENV infection, the approximate time to result for a given assay, specimen type required, when such a sample should be obtained, facilities required, and relative cost. An ideal assay would minimize the latter five criteria while remaining confirmatory. (WHO, 2009)

1.1.3.10. A Novel Method for DENV Diagnosis and Prognosis - Microsphere-based Immunoassay (MIA)

The microsphere immunoassay (MIA) and the Luminex™ platform are based on the merging of ELISA and flow cytometry laboratory assays. The first part of the assay involves the coupling of a capture molecule such as a protein antigen or short nucleotide sequence to a polystyrene microsphere. A target molecule within the sample then is captured and sandwiched with a detection molecule, and then subsequently labeled with streptavidin-PE. The second part of the assay is based on the specific wavelengths of two distinct lasers within the optical system of the platform. One laser is capable of reading a signal from streptavidin-PE, while the other is capable of identifying up to 100 distinct bead sets based on the ratio of two dyes contained within the beads. (Bio-Rad, 2010)

Regarding arboviral infections, the assay saw previous use in the serological detection of anti-WNV IgG and IgM antibodies in the presence of serum. The emergence of WNV in the Western Hemisphere led to the exploration of this platform as a potential advancement within current serological diagnostic and surveillance methods, and in this regard the CDC has approved its use (Johnson *et al*., 2007; Johnson *et al*., 2005). However, the assay is capable of detecting any number of serological markers provided that a method of capture and detection has been developed. In fact, this ability has led to the investigational use of the MIA not only in detecting WNV but additional arboviruses (Basile *et al*., 2013; Basile *et al*., 2010). The FLDOH-BPHL-Tampa was in the process of attempting to develop a single-well multiplexed assay capable of detecting antibody to WNV, SLEV, and eastern equine encephalitis virus, primarily for arboviral surveillance in sentinel chicken serum (Stark, *personal comm*.). The ability to multiplex has additionally led to the exploitation of the platform to detect both quantities of host-derived markers of infections such as chemokines and cytokines as well as virus specific markers (deOliveira-Pinto, *et al*., 2012; Gupta *et al*;, 2012; Thayan *et al*., 2010; Azizan *et al*., 2006; NE Reis *et al*., 2007; Shmitt *et al*., 2006). This, in turn, allows for the assay's usefulness to be expanded in its diagnostic capability as it now may be developed to detect both active and past infections. The ability to multiplex for target detection within the same well, on a level most diagnostic assays currently lack, only further enhances the flexibility and usefulness of the platform.

In order to have impact at the clinical level under this definition, the assay must be targeted at patients still in the early phases of disease, this likely rules-out the use of DENV antibody as a useful marker of infection in most cases (WHO, 2009). It has been recently suggested that the DENV nonstructural protein, NS1 may be useful in this regard, and an NS1 assay by ELISA is available from both Alere Inc. (Pan-Bio® Dengue Early ELISA #E-DEN02P) and Bio-Rad Laboratories, Inc. (Platelia Dengue NS1 EIA #72830). DENV NS1 is secreted from infected cells to detectable levels in serum as one of the earliest markers of infection and may persist into convalescence. It is a generally conserved protein among flaviviruses but has been found to contain both cross-reactive and serotype-specific epitopes among dengue viruses (Ding *et al*., 2011; Hu *et al*., 2011; Qiu *et al*., 2009; Xu *et al*., 2006; Falconar *et al*., 1994; Falconar and Young, 1991; Mason *et al*., 1990). In a report by Alcon *et al*., 2002 (French Guiana) where an ELISA using polyclonal antibodies directed againt DENV NS1 was used, NS1 detection was possible between days 1-9 of onset, may occur without detection of viral RNA and/or IgM, and showed no significant differences between primary and non-primary infections. The range of NS1 detection was 0.01-50µg/mL.

In 2006, the Platelia™ Dengue NS1 Ag microplate EIA (Bio-Rad, Hercules, CA) was used in the screening of 80,000 Puerto Rican blood donors. At the time of the FDA approval for the study, the test was already in use in approximately 40 countries around the world (Bio-Rad, 2010). Reports of the investigational use of commercially available DENV NS1 diagnotic assays in Brazil (Castro-Jorge *et al*., 2010) and Malaysia (Wang and Sekaran, 2010) showed favorable results when compared against traditional serological and virological methods with sensitivities of 95.9 and 76.76% and specificities of 81.1 and 98.31%, respectively. The former group used the Platelia™ Dengue NS1 EIA (Bio-Rad, Hercules, CA) and the latter, the SD Bioline Dengue Duo (Standard Diagnostics, Yongin-si, Rep. Korea). During the 2012-13 dengue epidemic in French Guiana, 3,347 suspected cases were subjected to both the Platelia™ and SD Bioline assays where the Platelia™ assay and RT-PCR were considered standard assays. The SD Bioline compared very favorably to both standards where it detected NS1 in 471 samples (14.1%) and the Platelia™ assay identified 14.2% samples as positive. The sensitivity and specificity of the SD Bioline assay were 87 and 92% respectively when compared to RT-PCR (Simmonet *et al*., 2017). In d.R.Q. Lima *et al*., 2010, two of the previously mentioned assays, the PanBio® ELISA

and the Platelia™ EIA, were compared against another immunoassay, the Dengue NS1 STRIP (Bio-Rad, Hercules, CA). The STRIP is an immunochromatographic test (ICT) similar to the previously mentioned SD Bioline Dengue Duo. While all obtained specificities near 100%, here they found the STRIP assay to have the highest sensitivity (89.6%), followed by the Platelia™ EIA at 83.6%, and the Pan-Bio® ELISA at 72.3%. However, concerns of the sensitivity of the Platelia™ assay arose in a study in Aracaju, Brazil where 58 of 119 EIA negative samples were later found to actually be DEN4 positive by confirmatory tests, alluding to an issue with the detection of secondary cases (Faria Sea *et al*., 2013). A subsequent report indicates that this drawback can be alleviated by heat-treating samples at 100°C for 5 minutes (d.R.Q. Lima *et al*., 2014). Previously in 2003, Koraka *et al*. developed a dot blot assay to detect DENV NS1 and found that acid treatment to release immune-complexed NS1 increased detection from 22/55 to 43/55 in their samples (compared to 32/55 in RT-PCR) additionally alluding to concerns with appropriate sample treatment prior to assaying. Gaikwad *et al*., 2017 took a similar approach to those detailed above in comparing a DENV NS1 ICT (Bhat BioScan Rapid Dengue NS1 Antigen, Bhat BioTech, Karnataka, India), DENV NS1 ELISA (RecombiELISA, CTK Biotech, San Diego, CA), and RT-PCR (here real-time). Compared to RT-PCR, the rapid ICT showed a sensitivity of 81.5% and specificity of 66.7% (concordance of 75.5%), whereas the ELISA showed a sensitivity of 89.9% and specificity of 100% (concordance of 94%). Another group (Mata *et al*., 2017) was interested in the effectiveness of using an ICT (BioEasy™ Dengue Eden Test, Standard Diagnostics, Yongin-si, Rep. Korea) in detecting DENV NS1 in whole-blood, point of care samples. In 120 positive dengue samples (combined RT-PCR and Platelia™ standards) this assay showed a sensitivity of 76.7% and specificity of 87% when read at 15m. Here, they also note that invalid results read at 15m should be read at 30m and that both sensitivity and specificity of the assay were higher for sera (82.2% and 100%). Additionally, DENV NS1 ELISAs derived from monoclonal antibody libraries capable of serotyping dengue virus NS1 in patient samples are under development, such as those reported in Austalia, via biopanning/phage-display (Lebani *et al*., 2017), as well as in China (Ding *et al*., 2011; Hu *et al*., 2011; Qiu *et al*., 2009; Xu *et al*., 2006) and Thailand (Puttikhunt *et al*., 2011). In all, these types of assays may represent the new paradigm for DENV diagnostics in many parts of the world.

It has also been well established that severe DENV illness is correlated with elevated levels of certain cytokines, chemokines, and other host-derived molecular markers in patient serum. For an assay to be of prognostic value, these host markers must be measurable in a significantly consistent yet differential pattern at an appropriate time during clinical presentation. A huge body of work has been compiled already, mainly in cell culture and mouse models of DENV infection, but a complete picture still remains elusive. Part of the problem stems from the lack of a true animal model for studying dengue and the difficulty with obtaining human serum/CSF samples for analysis. Regardless, a number of host markers, especially those that are pro-inflammatory in nature, may be useful for differentiating severity of dengue illness prior to development. IFN-α, IFN-γ, TNF-α, IL-2, 4, 6, 8, 10, 12, sIL-2R, macrophage migration inhibitory factor, CCL2/MCP-1, CXCL10/IP-10, IL-1β, elastase, sTNFR, RANTES, MIP-1α, MIP-1β, IL-1ra, sICAM-1, sVCAM-1, and VEGF have all been investigated and implicated as being elevated in DENV infections in one form or another. (Appanna *et al*., 2014; Rathakrishnan *et al*., 2014; deOliveira-Pinto, *et al*., 2012; Gupta *et al*;, 2012; Puerta-Guardo *et al*., 2012; Thayan *et al*., 2010; Azizan *et al*., 2009; Sun *et al*., 2009; Basu and Chaturvedi, 2008; Boonnak *et al*., 2008; Chen *et al*., 2008; Restrepo *et al*., 2008; Yen *et al*., 2008; Azizan *et al*., 2006; Halstead, 2007; Dong *et al*., 2007; NE Reis *et al*., 2007; Azizan *et al*., 2006; Fink *et al*., 2006; Shmitt *et al*., 2006 Shresta *et al*., 2006; Lin *et al*., 2005; Mangada and Rothman, 2005; Avila-Aguero *et al*., 2004; Dewi *et al*., 2004; Bosch *et al*., 2002; Suharti *et al*., 2002). Individual results are sometimes in direct conflict with other reports, paradoxically, given generally accepted, specific roles of these effector molecules in addition to the overarching model of DENV pathogenesis. This serves to point to the complexity in assessing serum markers for potential disease severity.

1.2. Statement of the Problem

Dengue is a problematic disease to manage at the clinical level. Until recently, the laboratory's capacity for diagnosing infections with DENV has been left wanting in many respects. Before any advances can be achieved regarding treatment, diagnostic methods capable of detecting disease at the earliest onset of symptoms are mandatory. Ideal diagnostic tools are sensitive, specific, fast, user-

friendly, and inexpensive. Diagnostic methods currently in use fulfill some, but not all, of these criteria. In cases where the disease is endemic and clinics are over-burdened with ill patients, a prognostic assay capable of allowing the proper management (triage) of patients is desired. Clinicians still rely upon traditional techniques when issuing prognoses where multiple tests may be requested using different techniques and equipment in order to establish a patient's risk of developing severe illness. This, in turn, taxes the already burdened health-care system and many patients that should be otherwise sent home are monitored unnecessarily. The development of an assay that can, in concert, diagnose infection with DENV and be of prognostic value could alleviate this burden. Recent advancements in the understanding of the clinical profiles of DENV-infected patients at the molecular level gives reason for hope. The MIA offers the possibility for achieving the task of concomitantly combining diagnostic capabilities via DENV NS1 detection with measuring host-derived markers capable of differentiating patients that will manifest uncomplicated dengue from those that will develop severe illness. This assay would be required to:

(1) diagnose infections by dengue virus with high sensitivity
(2) rule-out other infectious etiologies with high specificity
(3) have rapid turn-around time to result, ideally <24h after sample collection
(4) be cost-effective to those health-care systems that require it
(5) be capable of differentiating those patients that will progress to severe illness from those with uncomplicated dengue

Therefore, with the availability of the MIA platform to assess up to 100 different markers within the same well, it has the potential for becoming one of the most useful assays in the management of dengue patients. Determining which markers to include as a measure of potential disease severity at a time commensurate with patient presentation is a priority with respect to triage. Inclusion of a diagnostic marker indicative of DENV infection, here DENV NS1, is mandatory. This assay would simplify current patient care algorithms and reduce the workload at the clinical level as management of the patient would require only a single laboratory test in addition to the discretion of the attending physician. This study attempted to investigate the feasibility of such an approach.

1.3. Specific Aims

1. Evaluate cytokine production in DENV-infected HPMEC ST1.6R (endothelial) and u937 (monocyte) cell cultures and human sera using a 27-plex MIA cytokine assay.

2. Select relevant cytokines/chemokines for further analysis in MIA using DENV-infected human sera.

3. Evaluate monoclonal antibodies directed against DENV NS1 protein for use in MIA.

4. Use appropriate mAbs for detection of DENV NS1 in DENV-infected cell cultures and human sera via MIA.

5. Compare results of DENV NS1 MIA to a commercially available DENV NS1 ELISA.

CHAPTER 2. DETECTION AND QUANTIFICATION OF SELECT CYTOKINES AND CHEMOKINES PRODUCED IN *IN VITRO* MODELS OF DENV INFECTION VIA MIA

2.1. Introduction

As discussed in Chapter 1 (1.1.5.8.), several different types of cells are thought to play an important role in DENV pathogenesis. Monocytes and macrophages are thought to be targets of infection, especially during secondary (non-primary) infection, where they uptake non-neutralized virus when presented by professional antigen-presenting cells (APCs) and where antibody-dependent enhancement (ADE) of infection is thought to occur (Boonak *et* al., 2010; Honda *et al*., 2009; Boonak *et al*., 2008; Chen *et al*., 2008; Fink *et al*., 2006; Huang *et al*., 2006; Kontny *et al*., 1988). They additionally serve as potent immune effector cells during infection via production of numerous cytokines and chemokines either directly or via signaling cascades. Endothelial cells (ECs) within the vasculature are responsible for inducing plasma leakage that is a hallmark of DENV infection, especially during severe illness, either via direct damage, indirect damage, or the relaxation of gap junctions (Appanna *et al., 2014;* Azizan *et al*., 2009; Azizan *et al*., 2006; Peyrefitte *et al*., 2006; Dewi *et al*., 2004). Therefore, *in vitro* models that include cell lines derived from either type of cell have proven useful for studying certain aspects of DENV pathogenesis. In this study two cell lines, HPMEC ST1.6R (derived from human microvascular pulmonary endothelial cells) (Unger *et al*., 2002) and u937 (derived from human monocytes) (Sundstrom and Nilsson, 1976) were investigated for reasons that included ascertaining permissiveness of DENV infectivity and the resultant response of host markers to inoculation with DENV. The ultimate goal here was to obtain data that would be useful in developing immunological profiles and to supplement data that would be gathered from human sera as detailed in Chapter 3, in order to identify prognostic markers differentiating outcomes of DENV infection.

2.2. Studies in HPMEC ST1.6R Cell Line

2.2.1. Infection of DEN1 and DEN3 in HPMEC ST1.6R Cell Line, Confirmation of Infection via qRT-PCR, and Investigational Detection of DENV NS1 via ELISA

2.2.1.1. Methods

HPMEC ST1.6R cells were grown to 80-90% confluence in 6-well culture plates (Becton-Dickinson, East Rutherford, NJ, #3533934), overlaid with endothelial cell basal medium (EBM, Lonza, Basel, Switzerland, cat. CC-3121), and supplemented with endothelial cell growth medium (EGM SingleQuot™, Lonza, cat. CC-4133) on a substrate of 0.2% gelatin. Prior to inoculation, the cell culture medium was aspirated and the monolayer washed with HEPES. Volumes of 100uL of DEN1 and DEN3 (Table 2.1) at stock concentrations ($1.025x10^7$ and $6.666x10^6$ pfu/500µL, respectively) were inoculated onto the monolayer in duplicate along with mock (uninfected C636 cell culture supernatant) and control (EGM complete) wells in duplicate (i.e. one well, two plates) for each time point, control media were not included in 27-plex MIA. The plates were then incubated for two hours at 37°C, 5% CO_2 while being periodically rocked. After incubation, the inoculum was aspirated, the monolayer washed using EBSS, and 3mL EGM (supplemented EBM) added. Duplicate plates corresponding to time points 0, 2, 8, 24, 48, 96, 144, and 196h post-infection (p.i.) were then sealed and frozen at -70°C for later analysis via MIA, SYBR Green qRT-PCR, and investigational DENV NS1 detection via ELISA (the latter-most excluded samples at 2h and 8h). For the DENV NS1 ELISA (Inverness Medical, PanBio, Sinnamon Park, Queensland, Austalia, #E-DEN02P), DEN2-, DEN4-, WNV- (NY99), and SLEV- (TBH28) infected HPMEC ST1.6R samples were also included, from time points 0, 24, 48, 96, and 144h p.i. and diluted 1:2 with kit diluent (75µL/75µL) immediately prior to addition in ELISA. The DENV NS1 ELISA was then performed according to manufacturer's instructions. Index values for each sample were obtained first by dividing the absorbance values of each sample by the assay cut-off value. The cut-off value was obtained by multiplying the average absorbance values of the calibrators by the assay calibration factor. Index values

<0.9 were interpreted as negative for DENV NS1 detection, between 0.9 and 1.1 as equivocal, and >1.1 as positive results.

Table 2.1: Strains and titers of DENV1-4 used in for inoculating HPMEC ST1.6R and u937 cell-lines.

Serotype	Strain	Titer (pfu) 500µL
DEN1	Mochizuki Hawaii 1944	$1.025x10^7$
DEN2	New Guinea C	$1.450x10^7$
DEN3	H87 Philippines 1956	$6.666x10^6$
DEN4	H-241P Philippines 1956	$8.000x10^6$

2.2.1.2. Results

RNA detection of DEN1 and DEN3 of samples obtained from HPMEC supported only a low-level infection of HPMEC by DEN3 (Fig. 2.1) where all C_T values obtained were approximately=30. The C_T values obtained for DEN1-infected HPMEC were practically identical to those of mock-infected cultures at each time point, (appx.= 40) and taken as false positive results. The SYBR Green qRT-PCR that was in use during this stage of the study is especially prone to false positive signals at the extreme end of runs (Shu *et al*., 2003). Regarding DENV NS1 detection in HPMEC ST1.6R cultures, all DENV1-4 samples (at all time points p.i.) were positive as determined by ELISA (Table 2.1). Additionally, all negative control samples as well as all WNV- and SLEV-infected HPMEC cultures (at all time points) were found to be negative by ELISA. Interestingly, all DEN3 samples after 0h were found to have noticeably lower positive index values than those of the other three DENV serotypes.

Table 2.2: DENV NS1 detection via ELISA for DENV1-4-, WNV-, and SLEV-infected cell cultures of HPMEC ST1.6R cell line in addition to uninfected cell control cultures at selected time points post-inoculation (p.i.). DENV NS1 detection via ELISA was performed on DENV1-4-, WNV-, SLEV-, and mock-infected HPMEC cultures for samples taken at time points 0, 24, 48, 96, and 144h p.i. Note that all four DENV serotypes were positive for detection of NS1 at all time points whereas all samples at all time points for WNV, SLEV, and mock-infected controls were negative. Index values represent average absobance values of samples divided by the cut-off value. Samples with index values < 0.9 are negative, between 0.9 and 1.1 equivocal, and > 1.1 positive for DENV NS1 detection.

Sample	Index value	Result	Sample	Index value	Result
WNV 0h	0.30	Negative	**DEN4 24h**	6.60	**Positive**
WNV 24h	0.53	Negative	**Control 24h**	0.15	Negative
WNV 48h	0.87	Negative	**DEN1 48h**	6.56	**Positive**
WNV 96h	0.46	Negative	**DEN2 48h**	6.62	**Positive**
WNV 144h	0.87	Negative	**DEN3 48h**	5.41	**Positive**
SLEV 0h	0.71	Negative	**DEN4 48h**	6.57	**Positive**
SLEV 24h	0.25	Negative	**Control 48h**	0.20	Negative
SLEV 48h	0.34	Negative	**DEN1 96h**	6.40	**Positive**
SLEV 96h	0.41	Negative	**DEN2 96h**	6.47	**Positive**
SLEV 144h	0.33	Negative	**DEN3 96h**	4.22	**Positive**
DEN1 0h	5.91	**Positive**	**DEN4 96h**	6.38	**Positive**
DEN2 0h	6.60	**Positive**	**Control 96h**	0.70	Negative
DEN3 0h	5.20	**Positive**	**DEN1 144h**	6.62	**Positive**
DEN4 0h	6.55	**Positive**	**DEN2 144h**	6.60	**Positive**
Control 0h	0.22	Negative	**DEN3 144h**	3.88	**Positive**
DEN1 24h	6.53	**Positive**	**DEN4 144h**	5.37	**Positive**
DEN2 24h	6.60	**Positive**	**Control 144h**	0.26	Negative
DEN3 24h	1.84	**Positive**	HPMEC 1:2 (sample:kit diluent)		

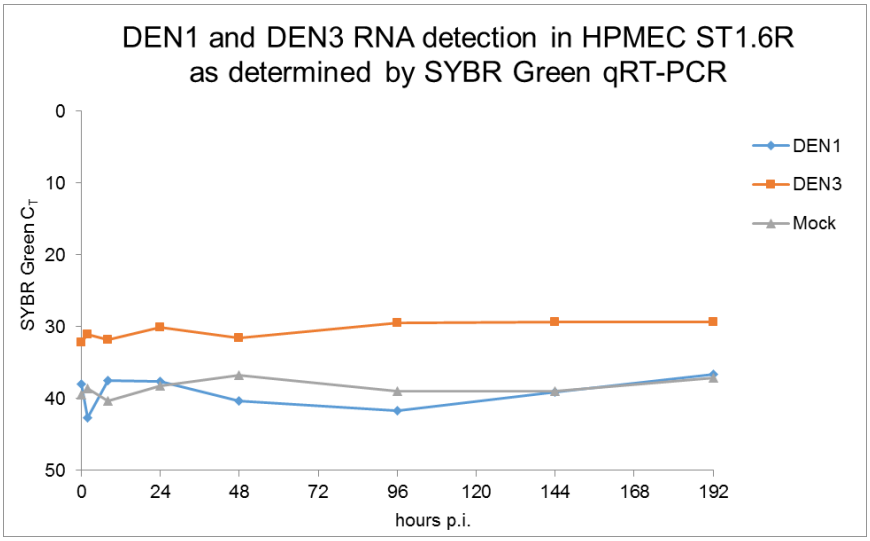

Fig. 2.1: RNA detection in DEN1, DEN3, and mock-infected HPMEC ST1.6R cell cultures via SYBR Green qRT-PCR at selected time points. The above figure illustrates C_T values obtained by SYBR Green-based qRT-PCR for DEN1, -3, and mock-infected HPMEC ST1.6R cell cultures at time points 0, 2, 8, 24, 48, 96, 144, and 192h post-inoculation (p.i.). Note that C_T values obtained for DEN3 tends to indicate a low-level detection of viral RNA/infection while the values for DEN1 tend to parallel those of mock-infected cultures indicating that a productive infection was not established. Plotted C_T values represent averages of triplicate wells. Uninfected controls were not included in qRT-PCR analysis.

2.2.2. Cytokine and Chemokine Analysis of DEN1- and DEN3-infected HPMEC ST1.6R Cell-line via 27-plex MIA

2.2.2.1. Methods

As stated above, duplicate plates containing DEN1-, DEN3-, and mock-infected HPMEC cell cultures were frozen at -70°C at time points 0, 2, 8, 24, 48, 96, 144, and 192h p.i. Upon preparation for qRT-PCR and 27-plex MIA analysis, these cultures were thawed and aliquots containing enough sample for each assay were collected. At this point, a modified version of the manufacturer's instructions were followed for the Bio-Plex Pro™ human cytokine 27-plex assay, (Bio-Rad #M500KCAF0Y) as outlined in the Bio-Plex Pro™ Assays: Cytokine, Chemokine, and Growth Factors Instuction Manual (Bio-Rad, Hercules, CA). The target analytes for this assay can be found in Table 2.2. The modification of this protocol occurred with a UV inactivation step after clarification of samples by centrifugation. For this UV inactivation step, each sample was added to an individual well of a sterile 96-well flat bottom plate containing enough volume for two reactions in MIA. These samples were then subjected to 10min UV exposure, the plate covered, subsequently wrapped in foil, and placed in a refrigerator at 4°C until samples were ready for use.

Table 2.3: Cytokine and chemokine target analytes of commercially available 27-plex MIA.

27-plex MIA cytokine kit (target analytes):			
IL-1β	IL-8	Eotaxin	MIP-1α
IL-1ra	IL-9	Basic FGF	MIP-1β
IL-2	IL-10	G-CSF	PDGF-BB
IL-4	IL-12(p70)	GM-CSF	RANTES
IL-5	IL-13	IFN-γ	TNF-α
IL-6	IL-15	IP-10	VEGF
IL-7	IL-17	MCP-1	

2.2.2.2. Results

Here, only 11 of the 27 analytes measured in the assay produced observable responses, 5 of which were later included in a 5-plex assay analyzing human sera (IFN-γ, IL-10, IP-10, GM-CSF, and

MCP-1) per our specific aims. Results that were of interest included profiles for IL-6, IL-8, VEGF, and MCP-1. Concentrations detected for IL-6 (Fig. 2.2 – top left) showed a peak at 2h, a subsequent waning until 48h, followed by a gradual increase in detection all the way through 192h for both DEN1 and DEN3 but not mock-infected HPMEC. Concentrations of IL-8 (Fig. 2.2 – top right) in DEN3-infected HPMEC were interesting for the fact that results were above the observable range of detection beginning at 96h. DEN1-, DEN3-, and mock-infected concentrations of VEGF in HPMEC (Fig. 2.2 – bottom left) all showed a similar profile until elevation occured in DEN3 beginning at 96h through 192h. Interestingly [VEGF] in both DEN1- and mock-infected cultures were practically identical until 192h when concentrations in DEN1 inoculated HPMEC rose to levels near those found for DEN3 (2478.38 versus 2587.79pg/mL, mock=1028.68pg/mL). Results for [MCP-1] in DEN1 and DEN3 (Fig. 2.2 – bottom right) showed similar profiles to those for IL-6, where an initial increase in detection at 2h was observed, followed by a waning until 24h, followed, here, by a more marked increase through 192h. The differences here though, were first, that concentrations of MCP-1 were nearly identical between serotypes until 24h and a marked increase in detection in DEN3 compared to DEN1 was only observed beginning at 48h. Additionally, unlike [IL-6] in mock-infected HPMEC where no, or nearly no, detection occurred over the course of infection, [MCP-1] in these cultures were found at elevated concentrations lying between those of DEN3 and DEN1 at 96h, 144h, and 192h.

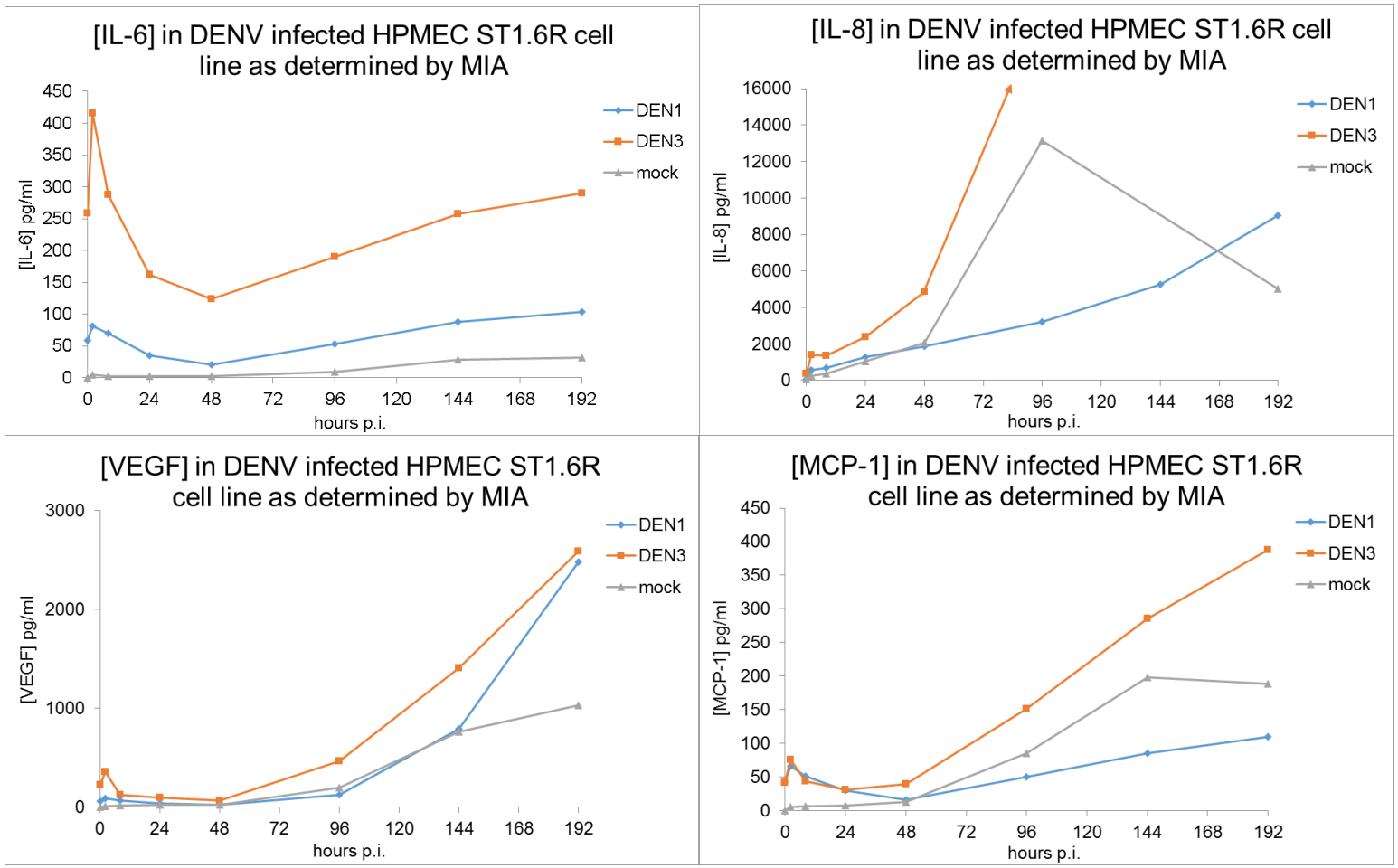

Fig. 2.2: Concentrations of IL-6, IL-8, VEGF, and MCP-1 (pg/mL) produced in either DEN1-, DEN3-, or mock-infected HPMEC ST1.6R cell-line at selected time points as determined by 27-plex MIA. The above figure illustrates the concentrations of IL-6 (top-left), IL-8 (top-right), VEGF (bottom-left), and MCP-1 (bottom-right) (pg/mL) as determined by 27-plex MIA for DEN1- (blue-diamond), DEN3- (orange-square), and mock-infected (gray-triangle) HPMEC ST1.6R cell cultures. These concentrations were measured for samples obtained at 0, 2, 8, 24, 48, 96, 144, and 192h post-inoculation (p.i.). Concentrations of IL-6 for each DEN1 and DEN3 showed a sharp peak at 2h, followed by waning in detection until 48h, and a gradual increase through 192h. IL-8 concentrations were above the observable range of the assay after 48h. DEN1 [VEGF] were nearly identical to those of mock-infected cultures until 192h when detection nearly matched that of DEN3. Concentrations of MCP-1 showed a similar profile to that of IL-6 but with a more dramatic increase through 192h. Each dot represents the average concentrations of individual samples ran in duplicate wells (DEN1 and-3) or single wells (mock).

2.2.3. Discussion

While endothelial cells of the vasculature are known to play an important role in the pathogenesis of dengue illness, their direct role in dengue virus infections remains unclear (Dewi *et al*., 2004). Certain models maintain that they are either not permissive to infection or play a largely indirect, but important role in pathogenesis, such as releasing immune effector molecules and reactions to DENV NS1 that cause either apoptosis or an increase in vascular permeability (Basu and Chaturvedi, 2008; Pang *et al*., 2007; Green *et al*., 2006; Lee *et al.,* 2006; Lin *et al*., 2005). Other models posit that infection of ECs with DENV plays a direct role in vascular leakage (Azizan *et* al., 2009; Azizan *et* al., 2006; Appanna *et al*., 2014; Ochoa, *et al*., 2009) and that direct infection can induce both production of IL-6 and IL-8 (Huang *et al*., 2000). In either case, all four of the analytes of interest here, IL-6, IL-8, VEGF, and MCP-1 have been found to play a role in response to DENV in ECs. The results of this *in vitro* study point to, at the very least, a degree of permissiveness to infection when comparing the qRT-PCR results of DEN1 versus DEN3 inoculated HPMEC cell cultures. It was our conclusion that no viral RNA was detected at any time point for DEN1-infected cultures while a moderate amount of viral RNA was detected at all time points for DEN3-infected cultures. As far as the investigational use of the DENV NS1 ELISA, the results here provided for some encouraging results. The fact that only DENV1-4-infected HPMEC samples were positive for NS1 detection would seem to rule out any cross-reactivity with culture-dervied proteins in addition to WNV and SLEV NS1. More in depth discussions of DENV NS1 assays such as this ELISA occur in Chapters 1 (1.1.5.10.) and 4. Results of the *in vitro* study using u937 DENV1-4-infected cultures were largely unacceptable and are not reported here with the following exceptions: similar attempts should use an MOI of at least 10 instead of 1, should be paired with ADE models of infection in this cell-line, and that treatment with DENV1-4 supported differential detection of RANTES, VEGF, and MCP-1 at 144h p.i. only.

It is important to note that the main purpose of performing 27-plex MIA screening here, was to determine if differential detection of the analytes measured occurred in DENV-infected cell culture models versus non-infected cultures, and if there were additional serotype-specific responses. The approach used here would not reveal much in a mechanistic way beyond these average differences in addition to

those that were time-dependent. This specific experiment provided useful results for only IL-6, IL-8, VEGF, and MCP-1 that would support their further investigation in similar experiments, which was the goal of using the screening approach described here. As stated above, each of these have been found to be elevated in both 'classic' and severe models of DENV *in vitro*. It is also important for the sake of developing immunological profiles that these results are also true *in vivo* such as elevated levels of IL-6 and IL-8 in DENV patients as reported in Avila-Aguero *et al*., 2004. Despite the methodological errors that are discussed above, we can report some positive results for 27-plex MIA of DENV-infected HPMEC. For instance, the results for IL-6, and to a lesser extent, MCP-1 exhibited the type of profiles that we were interested in, where clear differences were seen between sample types, both at individual time points, in addition to the duration of infection. If the results for [IL-8] could be reproduced, this would also be extremely interesting regarding the targeting of this analyte for DENV immunological profiling. It would also be valuable to determine where these values from 96h actually lie and this would require standards that would allow measurement. The profile for VEGF, where concentrations are dramatically higher at later time points also seems to support previous data. In all, this approach allows for the targeting of specific analytes, which will then allow for additional replicates, providing for robust statistical analyses. Although we were not primarily concerned with the biological reasons responsible for profiles in this study, rather only establishing differences, it is very enticing that these profiles can map, over time, what is happening *in vivo* and can serve to support the theories underpinning pathogenesis. For instance, it would be interesting to determine that if the dramatic increase in IL-8, a proinflammatory cytokine, that is seen here at around 96h in HPMEC is also seen *in vivo*, and if so, at what time during course of infection, and what the outcome of such a response would be (i.e. resolution of illness versus development of severe disease).

CHAPTER 3. IMMUNOLOGICAL PROFILES OF HUMAN SERA AS DETERMINED BY MICROSPHERE IMMUNOASSAY (MIA) DEMONSTRATES THAT IL-10, IP-10, AND MCP-1 LEVELS ARE ELEVATED IN ACUTE DENGUE INFECTIONS. AMBROSE, J.H., L.M. STARK, J.S. MATEUS, K.A. FITZPATRICK, AND A. AZIZAN. 2016. MICROBIOLOGY AND VIROLOGY. (INSTITUTE OF MICROBIOLOGY AND VIROLOGY; ASTANA, KAZAKHSTAN). 2(13):29-41

This manuscript was published in Microbiology and Virology. (Institute of Microbiology and Virology; Astana Kazakhsta) and was reprinted with permission (V. Berezin). It appears here in the form it was submitted, according to journal formatting with the exception of minor changes requested by the doctoral committee and appropriate headings for Sections, Tables, and Figures reflecting internal formatting of this document based on Chapter.

Immunological profiles of human sera as determined by microsphere immunoassay (MIA) demonstrates that IL-10, IP-10, and MCP-1 levels are elevated in acute dengue infections

Jason H. Ambrose[a,b], Lillian M. Stark[a,b], Jazmine S. Mateus[b,e], Kelly A. Fitzpatrick[c], and Azliyati Azizan[b,d]

[a]Global Health Department, College of Public Health, University of South Florida, 12901 Bruce B Downs Blvd., Tampa, FL, 33612, USA (ajason@health.usf.edu; lstark@health.usf.edu; aazizan@health.usf.edu)

[b]Bureau of Public Health Laboratories, Florida Department of Health, 3602 Spectrum Blvd. Tampa, FL, 33612, USA (Jason.Ambrose@flhealth.gov)

[c]Rickettsial Zoonoses Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA, 30329, USA (hwm8@cdc.gov)

[d]Nazarbayev University School of Medicine (NUSOM), 53 Kabanbay Batyr Ave., Astana, 010000, Republic of Kazakhstan (azliyati.azizan@nu.edu.kz)

[e]All Children's Hospital, Johns Hopkins Medicine, 501 6th Ave., St. Petersburg, FL, 33701, USA (jmateus@jhmi.edu)

Corresponding author:

Azliyati Azizan, PhD, MS

Nazarbayev University School of Medicine (NUSOM)

53 Kabanbay Batyr Ave.

Astana, Republic of Kazakhstan, 010000

Email: azliyati.azizan@nu.edu.kz

3.1. Abstract

Objectives: This study attempted to utilize the microsphere-based immunoassay (MIA) in order to ascertain immunological profiles of dengue virus (DENV)+ patient sera. It is hoped that developing profiles will alleviate patient management concerns and inform prognoses.

Methods: Two distinct sets of sera previously assayed for DENV were subjected to MIA. The first was assayed for 27 analytes and the second included 5 markers: GM-CSF, IFN-γ, IP-10, IL-10, and MCP-1.

Results: Four of 5 analytes (IFN-γ, IP-10, IL-10, MCP-1, but excluding GM-CSF) were observed at higher average concentrations in DENV PCR+ patient sera than those in DENV+ sera by ELISA and healthy individuals. Statistically significant differences were found for elevated levels of IL-10 in DENV PCR+ sera ($p=0.035$) and values for IP-10 ($p=0.093$) and MCP-1 ($p=0.058$) were of borderline significance. No significant differences were found for IFN-γ ($p=0.193$) or GM-CSF ($p=0.493$). These results point towards immunological profiles that may distinguish acutely ill DENV PCR+ patients from ELISA+ patients and healthy individuals.

Conclusions: The MIA is a useful rapid assay capable of producing meaningful immunological profiles of acutely ill DENV infected patients. To support the findings of this study, future studies should include larger sample sizes in addition to acute sera from severe DENV cases.

Keywords: dengue, DENV, cytokine, microsphere, immunological profile, Luminex

3.2. Background

Infection with dengue virus continues to be a worldwide threat to public health both in human and economic costs. In 2015, dengue is still the most significant arthropod-borne (arbo-) viral illness. Worldwide, an estimated 390 million infections occur annually where most cases go unreported; for

instance, in 2010 only 2.4 million cases were reported to WHO; where 96 million cases were actually thought to present clinically and the vast majority of infected individuals never seek medical attention. There is currently a global population of 3.9 billion at risk of acquiring dengue, according to WHO. Approximately 500,000 of these patients will progress to severe illness and require hospitalization, and around 2.5% of these cases will be fatal[1,2]. Additionally, in 2009-2010, dengue virus established its first recorded endemic transmission cycle in the state of Florida in over a half century, first within the Florida Keys during 2009-2010, followed by an additional, unrelated outbreak in Martin County in 2013[3]. There are four currently recognized serotypes of DENV and all four circulate in most endemic regions, whereas both outbreaks in Florida were caused by serotype 1 dengue virus.

Dengue is caused by one of four different serotypes of small RNA viruses in the family *Flaviviridae*, DENV1-4. The 5' and 3' ends of the DENV genome contain untranslated regions (UTRs) that vary in size according to serotype. The DENV genome's open reading frame encodes first, the three structural proteins, C, prM/M, and env, followed by 7 non-structural proteins and is translated as a single polyprotein that is processed and modified post-translationally[4]. The host response to dengue virus infection plays a large role in determining the resultant severity of illness and a laboratory test capable of predicting the course of illness would be of tremendous value to the clinic.

Lack of therapeutic treatment options coupled with a suitable vaccine on the market still not fully realized continue to offer challenges in attempts at controlling dengue. This leaves proper clinical management as the only option for reducing morbidity and mortality associated with cases of dengue. Infection with dengue leads to three stages of illness; the acute febrile phase, the critical phase, and finally followed by either recovery or worsening to severe illness. Severe illness may include any or all of the following: hemorrhage, shock, and/or death, but exhibits the common feature of plasma leakage due to an increase in vascular permeability. The febrile phase begins abruptly from 2-7 days after infection with non-specific symptoms. Most often, when patients present at the clinical level during this phase, those who may progress to severe illness and those that will experience uncomplicated illness are indistinguishable. The beginning of the critical phase usually coincides with defervescence on days 3-7 of illness, and lasts 24-48 hours. It is at this stage where 'warning signs' for development of severe illness often become apparent and certain patient criteria are evaluated for admitting and managing patients,

ranging from presence of fever to evidence of plasma leakage. At any stage prior to this, a laboratory assay that is able to identify and distinguish patients according to severity would be of tremendous value[5].

Clinicians still rely upon clinical and laboratory techniques such as measuring hematocrit and liver enzyme levels, in addition to evidence of pleural effusion, thrombocytopenia, petechiae, and/or hemorrhage, when determining patient prognoses. In a recent longitudinal study examining 504 patients with dengue-like symptoms, a number of these clinical findings were suitable for monitoring disease progression over time but unable to distinguish patients early during presentation[6]. It can be argued therefore, that clinicians lack an expedient platform for triaging patients with the greatest potential to develop severe illness. This, in turn taxes the already burdened health-care system; for instance, simply via the number of personnel that are required to interact with a single DENV patient. Also, many patients that should be otherwise sent home may be monitored unnecessarily.

Regarding the immune response of a given individual, the generation of normally beneficial inflammatory mediators in quantities that become damaging is generally thought to be a predominant mechanism involved with severe dengue pathology. This generation of a 'cytokine storm' is thought to be the ultimate factor in those that progress to severe illness. While it has been well established that severe DENV illness is correlated with elevated levels of certain cytokines, chemokines, and other host-derived molecular markers in patient serum, determining temporal profiles of these markers remains a key to informing prognoses. For an assay to be of prognostic value, these host markers must be measurable in a significant and consistent yet differential pattern at an appropriate time during clinical presentation when considering outcomes. While the sequence of cascades and the exact players involved have yet to be fully elucidated, TNF-α and IFN-γ seem to be two of the most important mediators and are often found to be elevated in severe illness [7,8]. Additionally, interleukins (ILs)-1β, 2, 4, 6, 8, 10, 12, macrophage migration inhibitory factor, granulocyte-monocyte colony stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP)-1 or CCL2, elastase, regulated and activated upon normal transcription expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, MIP-1β, interferon-γ inducible protein (IP)-10 or CXCL10, soluble intercellular adhesion molecule (sICAM-1), soluble vascular cell adhesion molecule (sVCAM)-1, and vascular endothelial growth factor (VEGF) have all been

implicated to play a role in severe illness and more appropriately illustrate the 'cytokine storm'[7-19]. However, individual results of studies are sometimes in direct conflict with other reports, given generally accepted models of the specific roles of these effector molecules as well as that of DENV pathogenesis. This serves to point to the complexity in assessing serum markers for predicting potential disease severity. Given the sheer number of cytokines, chemokines, and host-derived growth factors, and their myriad pathways as reported in the literature for dengue infections, the goal of identifying useful immunological profiles is certainly a daunting task. However, it is believed that a suitable minimum number of markers should become apparent that would allow for identifying immunological profiles capable of predicting the severity of illness in patients.

Determining immunological profiles would be most useful in locales where the disease is endemic and clinics are over-burdened with dengue patients. Here, a prognostic assay capable of allowing a more expedient method for the triage and proper management of retained patients is desired. As a platform capable of measuring multiple analytes representative of this 'cytokine storm', the microsphere immunoassay (MIA) and the Luminex™ platform may be useful in identifying host immune factors affected in response to infection by dengue virus. This information would be useful in developing immunological profiles of dengue patients that encompass the entire spectrum of clinical illness[20].

Therefore, the MIA has the potential of becoming one of the most useful assays in the management of dengue patients. Determining which markers to include as a measure of potential disease severity at a time commensurate with patient presentation is a desirable goal with respect to identifying dengue infected patients to triage and prioritize treatment. If a diagnostic marker indicative of DENV infection could be included within this assay, its value should only increase. This would also simplify current patient care algorithms and reduce the workload at the clinical level, as management of the patient would require only a single laboratory test in addition to the discretion of the attending physician. This study attempted to investigate the feasibility of such an approach; first by using a 27-plex MIA to screen for potential markers in human sera followed by a targeted 5-plex MIA.

3.3. Results and Discussion

3.3.1. Detection and Quantification of Select Cytokines and Chemokines Produced *in vivo* (DENV Positive Patient Sera) via 27-plex MIA

A group of 38 serum samples fitting one of five clinical criteria (see Methods 3.5.3.1.) were chosen for MIA analysis. The goal of using serum samples was to determine if the detection levels of the 27 analytes included for analysis (Table 3.1) resulted in observed profiles of differential detection for each of the individual analytes among these subsets of samples. Here, 19 of 27 analytes produced observable detection, including 4 of 5 analytes chosen for inclusion in a subsequent custom 5-plex assay, including IL-10, IFN-γ, IP-10, and MCP-1 but not GM-CSF (Figs. 3.1a-1d). The five analytes mentioned above were chosen for subsequent MIA analysis of sera, in part, due to the results of the 27-plex assay in addition to those of unpublished data from our *in vitro* studies of DENV infection using MIA. This unpublished data took into account the elevation of these markers in a temporal fashion as determined by MIA in DENV infected monocyte and endothelial cell culture-based systems.

Five analytes were examined in detail for the presence of significant differences between sera subsets. Neither IFN-γ [$F(4,28)=0.51$, $p=0.729$] nor MCP-1 [$F(4,28)=0.09$, $p=0.985$] analyte results indicated that sample groups were distinct in the production of these host markers. The smallest p-values, also lacking a statistically significant difference, were found for the IL-10 [$F(4,28)=1.20$, $p=0.333$] and IP-10 [$F(4,28)=1.29$, $p=0.298$] analytes, where F is the ratio of the between groups to within groups mean square for each test.

For the IL-10 analyte, each sera subset had multiple undetected analyte concentrations, *i.e.* zero values, with all measurements below 50pg/mL. Interquartile ranges of the sample subsets were determined for each analyte to denote the range of concentrations falling within the middle 50% of values (calculated as the difference between the first and third quartiles). These are denoted by the shaded box regions of Fig. 3.1a-d. Acute sera positive for DENV had the largest interquartile range (IQR=9.74pg/mL) of all sera subsets for this analyte, including DENV negative sera (IQR=0.17pg/mL). The widest overall range of concentrations (40.30pg/mL), as well as the greatest mean (μ=7.53pg/mL) and standard

deviation (σ=13.56pg/mL), were also found in the acute DENV positive sera group. All subsets had similar distributions and median values, ranging from 0pg/mL to 0.34pg/mL.

IFN-γ analyte concentrations were similar across all 5 sera subsets, with all concentrations under 150pg/mL and few zero values in any group. Interquartile ranges were comparable among sample groups, with the widest (IQR=43.72pg/mL) and narrowest (IQR=17.41pg/mL) ranges calculated in DENV negative and convalescent WNV/Flavivirus positive sera, respectively. Mean concentrations were consistent across groups, around 43pg/mL, except where the convalescent DENV positive subset produced a lower mean of 22.47pg/mL. Standard deviations were ~18pg/mL for most groups, with the DENV negative sera subset standard deviation (σ=63.54pg/mL) exceeding its mean (μ=42.06pg/mL), while the acute DENV positive group standard deviation nearly matched its mean (μ=43.05pg/mL, σ=35.65pg/mL).

IP-10 analyte data contained nearly all detectable concentrations, with the greatest sample group interquartile range observed in the acute DENV positive group of 8971.42pg/mL. Six sera (~43%) from this set had concentrations above detectable limits, which was not observed for any other subset or analyte. These values were replaced with 9000pg/mL, in order to facilitate calculations. The interquartile range of the DENV negative subset was 4126.63pg/mL, while the remaining groups had interquartile ranges below 1500pg/mL. However, DENV negative sera had the highest median IP-10 concentration of 2216.60pg/mL, followed by acute DENV positive sera (median=1336.21pg/mL). Medians under 975pg/mL were found for the other sets of sera. Each group mean and standard deviation were comparable within each subset, *i.e.* DENV negative (μ=2279.55pg/mL, σ=3107.59pg/mL), but not across groups, except for convalescent DENV positive and DENV negative sera.

Values for the MCP-1 analyte were similar to those observed for IFN-γ. All concentrations were below 250pg/mL and only one undetectable value was obtained. The smallest interquartile range was 36.56pg/mL (convalescent WNV/Flavi positive) and was analogous to those calculated for the other sera subsets, which were in the forties. Means (~47pg/ml) and standard deviations (~57pg/mL) were similar within and across sera subsets. Medians were comparable across sample groups, ranging from 16.28pg/mL to 36.59pg/mL.

Differences between sera sample subsets tested by 27-plex MIA were not statistically significant for any of these analytes by ANOVA, additionally GM-CSF analyte concentrations were undetectable. Despite the absence of statistical significance in the differential detection of these 4 analytes and GM-CSF, all 5 candidate analytes were included in the subsequent experiment detailed below. This was due in part, to their expression compared to that of the other 22 analytes among the sera samples here, our group's unpublished *in vitro* data as mentioned above, and finally due to their presumed importance in the pathogenesis of dengue[7-9,12,13,16-18].

3.3.2. Detection and Quantification of Select Cytokines and Chemokines of Selected Serum Samples via 5-plex MIA

A subsequent group of 24 serum samples fitting one of four criteria (see Methods 3.5.4.1.) were chosen for MIA analysis in a custom 5-plex assay; these analytes were chosen, in part, due to the results of the previous set, in addition to unpublished data as mentioned above. The goal of using serum samples here was to determine if the detection levels of the 5 chosen analytes resulted in observed profiles of differential detection for each of the cytokines/chemokines among the included subsets of sera as detailed in Methods. Each analyte chosen for inclusion in the 5-plex assay produced observable detection levels (Figs. 3.2-3.6) within at least two of the four sample subsets. Results here were also compared to the detection levels of these analytes via MIA in a separate study using healthy individuals (shaded area of Figs. 3.2a-6a)[21]. The inclusion of DENV qRT-PCR positive samples in this assay proved to support the selection of these 5 analytes when compared to DENV ELISA positive/PCR negative samples and negative sera (with the exception of GM-CSF) as well as healthy subjects.

In all, 4 of the 5 analytes (IL-10, IFN-γ, IP-10, and MCP-1) discussed here were detected in all 5 sera groups of the first set of samples, as well as all 4 of the groups of sera in the second set of samples. GM-CSF, which was not detected in any of the sera (below the limit of detection) in the initial group of sera was only detected in two groups of samples in this second set, the first being the DENV qRT-PCR+ samples. DENV negative samples obtained from a serosurvey that were included due to their presumably healthy status, were also found to have GM-CSF present. Perhaps most importantly, each of

the 5 analytes were detected in markedly higher average concentrations in DENV qRT-PCR+ samples as opposed to samples that were only serologically positive for anti-DENV IgG (Table 3.2), including GM-CSF. Detection of viral RNA in patients indicates current infection and therefore presumably acutely ill patients, an important indicator for differentiating immunological profiles between groups of patients.

Concentrations of each analyte were approximately ten times higher (or more) on average for DENV PCR+ sera than any other category with few exceptions (Table 3.2). GM-CSF was on average found in higher concentrations for DENV negative patients from the serosurvey than those from DENV PCR+ patients. IFN-γ was found only 2-5 times higher on average in PCR+ sera than all but two other categories (DENV ELISA+ convalescent sera and clinical negative sera). Clinical negative sera were also found to be only approximately 2 times lower on average for IP-10 and 3 times lower for MCP-1 than DENV PCR+ sera. The final exception was that MCP-1 was found to be produced on average 2.5 times higher in DENV+ PCR sera than samples positive for DENV IgG only.

It must be noted, however, that the range of values obtained for the largest group (PCR positive sera) does skew these average concentration value comparisons in some instances. For instance, while GM-CSF was found, on average, in higher concentrations for DENV negative sera from the serosurvey (n=3), the highest actual value obtained from these samples was 21.41pg/mL, whereas the highest value obtained from the DENV PCR positive samples (n=14) was 96.7pg/mL (Table 3.2). Possible reasons for this discrepancy in GM-CSF detection are discussed below. Samples that were found to be above the range of detection were an issue, particularly for IP-10 in acute DENV positive ELISA sera (1st set) and MCP-1 (2nd set) where n=6 and n=1 sample(s), respectively, were out of range and so the reported average concentrations here are artificially skewed lower. Additionally, markedly higher concentrations of IFN-γ were produced in only 2 of 14 DENV qRT-PCR+ samples yet the average of these values still lies above the range for that of presumably healthy individuals in Kleiner *et al*[21]. where subjects were ruled out if reporting any acute or chronic condition or were on medication.

While the above problems involved with this study are factual, nonetheless this study has established the framework of an immunological profile for uncomplicated dengue compared to healthy individuals using these five markers as none of the analytes on average fall within the range of average production by a healthy individual. Concentrations of IL-10, IFN-γ, IP-10, and MCP-1 were found to be

higher, on average, in DENV PCR+ patients than healthy individuals, while the concentration of GM-CSF was found to be lower on average in the former than the latter. Furthermore, while the variance in concentrations remains for 4 of the 5 analytes (excluding GM-CSF) between any given individual, it is also important to note that most of these observed concentrations are above the range produced by healthy individuals.

Figures (3.2-3.6) illustrate the concentrations (pg/mL) of five analytes (IL-10, IP-10, MCP-1, IFN-γ, and GM-CSF) as determined by 5-plex MIA. The shaded area in each figure represents the range of concentrations obtained for each analyte when also measured by MIA in healthy individuals within a separate study (Figs. 3.2a-3.6a)[21]. Two sets of sera were compared; DENV positive and DENV negative. While diagnostic status was determined by both qRT-PCR and ELISA, the DENV positive group included only samples detected by PCR for analysis.

Although statistically significant p-values were not obtained with 27-plex MIA screening, results of the 5-plex MIA analysis (Fig. 3.2-3.6) suggest that significant differences in the concentrations of at least 3 of the 5 analytes may occur in acutely DENV positive sera. Comparison of IL-10 concentrations in positive versus negative sera (Fig. 3.2a-b), evidenced a statistically significant elevation of the IL-10 analyte [$p=0.035$, $t(17)=1.94$] in DENV+ specimens. Borderline p-values were calculated for concentrations of IP-10 [$p=0.093$, $t(17)=1.38$] (Fig. 3.3a-b) and MCP-1 [$p=0.058$, $t(15)=1.67$] (Fig. 3.4a-b) when comparing DENV positive and negative samples.

The p-values illustrating differences in IFN-γ [$p=0.193$, $t(17)=0.89$] (Fig. 3.5a-b) and GM-CSF [$p=0.493$, $t(17)=0.02$] (Fig. 3.6a-b) analyte concentrations by 5-plex MIA did not suggest statistically significant differences between diagnostic groups. This implies that although the presence of these analytes may be detected by MIA, similar concentrations might be found in DENV positive specimens as would be identified in DENV negative samples. However, a range of expected concentrations cannot be determined with data from this study, due to the small sample size of sera, particularly in DENV negative sera tested (n=5). It is important to note that small sample sizes may also affect the reproducibility of these results, for both negligible and borderline ($p>0.05$) as well as statistically significant ($p<0.05$) differences.

3.3.3. Developing Immunological Profiles for DENV-infected Individuals

While it may be interesting, or even useful, to compare detection of a specific analyte among two or three groups of sample types, it must be remembered that the most important long-term goal of the study is to develop a cytokine/chemokine profile for DENV infections that would guide development of the MIA for prognostic purposes. It may at first glance be surprising that IP-10 was detected at the highest concentrations for DENV qRT-PCR+ and DENV negative clinical samples (2nd sample set), but it is likely that the latter group were patients who were experiencing some kind of illness as opposed to samples from the serosurvey, given the clinical origin of the samples. On the other hand, GM-CSF was only detected in DENV qRT-PCR+ samples and DENV negative samples from the serosurvey, but it is not known what other characteristics of these individuals in the survey were present at time of collection outside of specific selection criteria. While the average response of each analyte was detected in a markedly higher fashion for DENV qRT-PCR+ samples, again, this does not serve to inform prognosis, even retroactively, as all patients resulted in uncomplicated illness. Rather, the important aspect to consider here is that these results point towards immunological profiles distinguishing acutely ill patients with uncomplicated dengue from convalescent patients and healthy individuals. While these results may in fact illustrate immunological profiles that prove beneficial to resolution of disease, unfortunately, the lack of available clinical serum samples from patients that developed severe DENV illness abrogates any information that would be useful for determining prognoses. The true test, and thus the true utility of the assay, will be to analyze a set of analytes (cytokines/chemokines) that are differentially expressed, at the time of clinical presentation, from those patients that will exhibit less severe manifestations of DENV illness from those that will experience severe illness. Hopefully, some or all of these markers can lead to that particular need; this will be investigated further in future studies.

Profiling disease outcome or novel intervention in DENV infections has been *en vogue* recently, including those determined by similar MIAs. *In vitro* immune responses were measured where potential therapeutics such as dexamethasone[14] and vitamin D3[17] were shown to downregulate the inflammatory response in dengue infected cell lines, including decreased levels of IL-10, where the former study was performed by MIA. The temporal profile of VEGF production in the HPMEC ST1.6R cell-line as

determined by MIA in a study by Azizan *et al*[10]. further illustrates the utility of the platform and this approach in selecting markers detected at differential levels at a suitable time during clinical presentation of dengue patients. Another study showed that both IP-10 (at two distinct levels) and MCP-1 were found to be elevated in dengue patients via MIA versus controls; this group also considered the presence or absence of both fever and arthralgia as co-variables[13]. In NI Reis *et al*[17]. IL-10 was detected at significantly higher concentrations in DENV infected monocytes than controls (which remained near undetectable levels). However, as these concentrations were obtained via infection of a single cell-type in an *in vitro* model, the specific values obtained cannot be directly compared to values obtained in our study. A study by Appanna *et al*[12]. showed via MIA that IFN-γ is downregulated in uncomplicated dengue and upregulated in severe dengue when compared to healthy individuals. Healthy controls exhibited a mean serum concentration of 157.2pg/mL, those exhibiting uncomplicated dengue had a peak average of 89.8pg/mL at days 4-6 of illness, and severe dengue patients showed a peak mean of 192pg/mL at days 2-3 post-onset (and the lowest mean here was 184.8pg/mL at days 7 or above during illness). The mean concentration of 241.08pg/mL obtained for DENV PCR+ for IFN-γ in our study exceeded all of the mean concentrations obtained in the aforementioned study. This group also showed that the temporal profile of IP-10 production is highest in acute cases of both mild and severe dengue and wanes over time, where production is much higher in both when compared to healthy controls (but is highest in severe cases). Healthy controls exhibited a mean concentration of 1114.7pg/mL, uncomplicated dengue patients had detectable average levels of IP-10 ranging from 46,967-20,505pg/mL in a waning fashion during course of illness. For DENV PCR+ individuals in our study, the mean concentration of IP-10 was 19115pg/mL, far from the peak of DENV+ patients in the aforementioned study, but also far from their healthy individuals. Likewise, severe dengue patients showed a similar but more magnified profile where the range of peak means of 71569pg/mL in those at days 2-3 of illness decreased to 24852pg/mL. MCP-1 production showed a similar profile but where differences between non-severe and severe dengue were marginal. Healthy controls evidenced mean MCP-1 concentrations of 84.1pg/mL, while the profiles of both uncomplicated dengue (397.6-117.1pg/mL) and severe dengue patients (400.4-152.3pg/mL) showed a similar pattern of decreasing detection from days 2-3 to 7 and beyond onset of illness. In our study, the mean concentration of MCP-1 production in DENV PCR+

individuals was 506pg/mL and, like IL-10, exceeds all average detectable levels for MCP-1 production in the study mentioned. The factor for these differences between studies may be due to population differences (*i.e.* those in endemic areas versus travel-associated cases). Finally, in a study by Yeo *et al*[22]. a large number of host markers, including TNF-α and IL-10, were found to be downregulated in asymptomatic, dengue-infected individuals along with upregulation of a few markers such as RANTES, evidencing further the diverse profiles possibly produced during the course of infection. Although this study used gene regulation as the determining factor, MIA may have easily been substituted in its place for measuring concentrations of these markers instead. Taken together, these studies as well as the one reported here, further support the utility of the MIA in developing immunological profiles for DENV infections.

Furthermore, while the aims of this study did not explicitly include developing a more complete model of DENV pathogenesis, mechanistically speaking, some additional observations of these results are worth mentioning due to the specifics of certain profiles of markers that were obtained. For instance, upregulation of IFN-γ, shown elsewhere to be at highest concentrations during the febrile phase, induces production of IP-10 (CXCL10). In turn, the *in vivo* polymeric form of IP-10 and its cognate receptor CXCR3 have been shown to be important for leukocyte/T-cell migration from the endothelium to sites of inflammation in the tissue[23]. IL-10 has been shown to be elevated in production all the way through to defervescence and its elevation is even higher in severe illness[24]. The literature modeling the pathogenesis of DENV therefore supports both the inclusion of these analytes in addition to the profiles observed in acute sera (DENV PCR+) in this study. It will be interesting to determine the biological relevance of the markers chosen here in further establishing a more complete model of DENV pathogenesis. These results therefore, provide the groundwork for future studies with the hope of providing some sort of contribution in understanding the complex biological mechanisms involved in the various manifestations of this disease.

3.4. Conclusions

In conclusion, the detection of differential levels of all 5 cytokines and chemokines (GM-CSF, IL-10, IFN-γ, IP-10, and MCP-1) via MIA seems to point to clear differences in the immunological profiles between acutely infected and those of convalescent DENV positive patients as well as DENV negative individuals. These differences are even more pronounced when compared to healthy individuals. Furthermore, statistical analyses support or at least point towards significant differences for at least 3 of the 5 analytes (IL-10, IP-10, MCP-1) included in the 5-plex assay when comparing all DENV+ samples versus all negative sera. Thus, we conclude that at least 3 of the 5 analytes chosen show promise for determining useful immunological profiles specific to dengue. Repeating this study with a larger sample size would likely strengthen p-values, providing more compelling evidence that the concentrations of IL-10 detected via MIA are higher in sera testing DENV+ by PCR and/or ELISA and/or return converse conclusions about MCP-1 and IP-10 analytes. Similarly, increasing sample size may also produce more statistically significant observed profiles for GM-CSF and IFN-γ. Additionally, quantification of expected normal and abnormal ranges of the five analytes in DENV positive and negative samples may also be possible with more sera subset testing. Future goals include the confirmation of study findings utilizing greater sample numbers, the inclusion of acute sera obtained from severe dengue infections, selecting appropriate analytes to differentiate non-severe from severe cases of DENV prior to progression, and selecting an appropriate number of analytes for assay feasibility.

3.5. Methods

3.5.1. Ethics Statement

The removal of identifiers and blinding of previously collected diagnostic samples resulted in this study being determined as not meeting the definition of human research activities and thus IRB exempt under US 45 CFR 46.101(4). This ruling was determined by the University of South Florida IRB.

3.5.2. Dengue Diagnostic Status of Samples Selected for Study

The first set of serum samples selected for inclusion in the 27-plex MIA (n=38) were previously assayed at the Bureau of Public Health Laboratories (BPHL)-Tampa for the presence of arboviral antibodies by IgG and IgM-MAC ELISA, including DENV, WNV, and SLEV, via protocols adapted from CDC (Ft. Collins, CO). The second set of sera, regardless of source (see below), were previously assayed for DENV infection by any combination of 3 assays. Each sample may have had diagnostic status for DENV established by any combination of a CDC approved DENV TaqMan-based qRT-PCR (San Juan, PR) and/or the previously mentioned ELISAs.

3.5.3. Detection and Quantification of Select Cytokines and Chemokines Produced *in vivo* (DENV Positive Patient Sera) via 27-plex MIA

3.5.3.1. Sample Selection

A series of 38 de-identified serum samples provided by BPHL-Tampa were selected based on specific clinical and serological criteria and subsequently blinded and randomized prior to use in this study. Acute DENV positive sera (S1) would indicate either detection of anti-DENV IgM in a sample or that of a four-fold increase in anti-DENV IgG titers from acute sera (S1) to convalescent sera (S2). BPHL-Tampa may also serologically detect either WNV or SLEV antibodies in patient sera but due to cross-reactivity within the assay the etiological agents are not always clearly identified. These samples are termed here as either non-DENV flavivirus (FLAVI) positive or negative. Past flaviviral infections included samples where IgG titers were not indicative of a recent infection. The following criteria were used for selection and based on ELISA results: 1. **acute positive sera (S1) for dengue** (DENV+, n=14), **non-dengue flavivirus** (FLAVI+, n=4) 2. **convalescent positive sera (S2) for DENV+** (n=7) 3. **convalescent (S2, S3) or past flavivirus positive of indeterminate onset** (FLAVI+, n=4) 4. **arbovirus negative** (n=4). One DENV+ sample from each of the S1 and S2 subsets were positive via RT-PCR.

3.5.3.2. Cytokine and Chemokine Analysis of Selected Patient Sera via 27-plex MIA

Thirty-seven samples of sera were ran in duplicate wells and concentrations determined via BioPlex Manager 5.0 software (Bio-Rad, Hercules, CA). Concentrations were reported as the average of the resultant two values when compared to standard curves generated against known values of the analytes that were provided for in the kit. The analytes included in the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad #M500KCAF0Y) for MIA are listed in Table 3.1. All other steps were followed per manufacturer's instructions[20].

3.5.3.3. Statistical Analysis of Results Obtained for Selected Patient Sera Measured via 27-plex MIA

One-way, Between Groups ANOVA in Microsoft Excel 2013 was performed to compare sera subset analyte concentrations by 27-plex MIA. The same software was used to generate boxplots of concentrations for sample subsets, which were created to illustrate variability, range, and central value across sera subsets.

3.5.4. Detection and Quantification of Select Cytokines and Chemokines of Selected Serum Samples via 5-plex MIA

3.5.4.1. Sample Selection

A series of 24 de-identified serum samples provided by the BPHL-Tampa Lab were obtained either from the clinical archive or by way of a serosurvey conducted in Martin County during the course of a DENV outbreak in 2013. The sera were selected based on specific molecular and serological criteria and subsequently blinded and randomized prior to use in this study. Sera that were positive via qRT-PCR for DENV indicate sera that contained DENV viral RNA and therefore an active or very recently active infection. Sera that were DENV positive for IgG only indicate samples that were found to have detectable anti-DENV IgG but not DENV RNA or anti-DENV IgM. These may include samples from

actively infected secondary infections, convalescent patients, or those that may have been infected in the past. The DENV negative samples here fall under one of two criteria, in both cases the samples were negative for DENV but came from either the BPHL-Tampa clinical sample archive or from a serosurvey conducted in Martin County during a DENV outbreak. The following criteria were used for selection and based on either PCR or ELISA results, or both: 1. **TaqMan-based qRT-PCR DENV+** (n=14) 2. **DENV+, IgG only** (n=4) 3. **DENV negative** (BPHL-Tampa clinical archive, n=2) 4. **DENV negative** (Martin County serosurvey, n=4).

3.5.4.2. Cytokine and Chemokine Analysis of Selected Serum Samples via 5-plex MIA

Twenty-four samples of sera were ran in duplicate wells and analyte concentrations as determined via BioPlex Manager 5.0 software were reported as the average of the resultant two values. These concentrations were extrapolated from standard curves generated against known values of the analytes. The 5 analytes included in this 5-plex custom MIA are listed in bold in Table 3.1 and included the bead sets for detecting: IL-10 (Bio-Rad #171-B5010M), GM-CSF (Bio-Rad #171-B5018M), IFN-γ (Bio-Rad #171-B5019M), IP-10 (Bio-Rad #171-B5020M), and MCP-1 (Bio-Rad #171-B5021M). The 5-plex MIA was performed per manufacturer's instructions[20]. Reagents included in the 27-plex assay kit not included with the 5-plex custom MIA kit were provided via the Bio-Plex Pro Human group I Cytokine standards (Bio-Rad #171-D50001) and the BioPlex Pro™ reagent kit (Bio-Rad #171-304070). The latter included antibody diluent, standard diluent, sample diluent, assay and wash buffers, streptavidin-PE, 1 x 96 well filter plate, and plate sealing tape.

3.5.4.3. Statistical Analysis of Results Obtained for Selected Patient Sera Measured via 5-plex MIA

The Two-Sample, t-Test Assuming Unequal Variances was used to statistically assess differences between DENV positive and negative sera subsets in the 5-plex assay. Microsoft Excel 2013 was used to perform the analysis and create graphical displays of the data. Due to the limited availability of suitable specimens, analyte concentrations in biologically similar subsets (DENV negative sera) were

aggregated during analysis to strengthen the reliability and reproducibility of the analysis; *i.e.* DENV negative (DOH) n=2 and DENV negative (serosurvey) n=3 were analyzed as one group (n=5). DENV+ sera by PCR were incorporated into the analysis, while sera found to be DENV positive by IgG were excluded. The two-fold rationale behind this omission can be attributed to the inadequate sample size of the IgG DENV+ group (n=2) and the increased likelihood of a familywise error occurring as more comparison groups are included in an analysis.

3.6. Availability of Supporting Data

The datasets supporting the conclusions of this article are available in the Open Science Framework (OSF) repository at https://osf.io with the persistent, external identifiers doi:10.17605/OSF.IO/SRB6Dand ark:c7605/osf.io/srb6d.

3.7. References

[1]. World Health Organization (WHO). Dengue and severe dengue. 2015. http://www.who.int/mediacentre/factsheets/fs117/en/. Accessed 15 Aug 2015.

[2]. Gullard A. Burden of dengue fever is higher than previously thought. BMJ. 2013; 347:f6280; doi:10.1136/bmj.f6280.

[3]. Florida Health. Dengue Fever- dengue occurrence in Florida. 2015. http://www.floridahealth.gov/diseases-and-conditions/dengue/. Accessed 18 August 2015.

[4]. Beasley DWC, Barrett ADT. The infectious agent. In: Halstead SB, editor. Dengue. London: Imperial College Press; 2010. p. 29-73.

[5]. World Health Organization (WHO). Dengue: guidelines for diagnosis, treatment, prevention and control- new edition. Geneva: WHO Press; 2009.

[6]. Rathakrishnan A, Klekamp B, Wang SM, Komarasamy TV, Natkunam SK, Sathar J, Azizan A, Sanchez-Anguiano A, Manikam R, Sekaran SD. Clinical and immunological markers of dengue progression in a study cohort from a hyperendemic area in Malaysia. PLoS One. 2014; doi:10.1371/journal.pone.0092021.

[7]. Basu A, Chaturvedi UC. 2008. Vascular endothelium: the battlefield of dengue viruses. FEMS Immunol. Med. Microbiol. **1:**1-13.

[8]. Fink J, Gu F, Vasudevan SG. Role of T-cells, cytokines and antibody in dengue fever and dengue haemorrhagic fever. Rev. Med. Virol. 2006; 16:263-75.

[9]. Halstead SB. Dengue. Lancet. 2007; 370:1644-52.

[10]. Azizan A, Fitzpatrick K, Signarovitz A, Tanner R, Hernandez H, Stark L, Sweat M. Profile of time-dependent VEGF upregulation in human pulmonary endothelial cells, HPMEC-ST1.6R infected with DENV-1, -2, -3, and -4 viruses. Virol. J. 2009; doi:10.1186/1743-422X-6-49.

[11]. Azizan A, Sweat J, Espino C, Gemmer J, Stark LM, Kazanis D. 2006. Differential proinflammatory and angiogenesis-specific cytokine production in human pulmonary endothelial cells, HPMEC-STR1.6R infected with dengue-2 and dengue-3 virus. J. Viro. Met. 2006; 138:211-17.

[12]. Appanna R, Wang SM, Ponnampalavanar SA, Lum LCS, Sekaran SD. Cytokine factors present in dengue patient sera induces alterations of junctional proteins in human endothelial cells. Am. J. Trop. Med. Hyg. 2012; 87(5):936-42.

[13]. de Oliveira-Pinto LM, Gandini M, Picinini Freitas L, Mendonça Siqueira M, Ferreira Marinho C, Setúbal S, Fernandes Kubelka C, Gonçalves Cruz O, de Oliveira SA. Profile of circulating levels of IL-1Ra, CXCL10/IP-10, CCL4/MIP-1β, and CCL2/MCP-1 in dengue fever and parvovirosis. Mem. Inst. Oswaldo Cruz. 2012; 107(1):48-56.

[14]. Puerta-Guardo H, De la Cruz Hernández SI, Rosales VH, Ludert JE, del Angel RM. The 1α,25-dihydroxy-vitamin D3 reduces dengue virus infection in human myelomonocyte (U937) and hepatic (Huh-7) cell lines and cytokine production in the infected monocytes. Antiviral Res. 2012; 94:57-61.

[15]. Boonnak K, Slike BM, Burgess TH, Mason RM, Wu SJ, Sun P, Porter K, Rudiman IF, Yuwono D, Puthavathana P, Marovich MA. Role of dendritic cells in antibody dependent enhancement of dengue infection. J. Virol. 2008; 82(8):3939-51.

[16]. Restrepo BN, Isaza DM, Salazar CL, Ramirez R, Ospina M, Alvarez LG. Serum levels of interleukin-6, tumor necrosis factor-alpha and interferon-gama in infants with and without dengue. Revista da Sociedade Brasileira de Medicina Tropical. 2008; 41(1):6-10.

[17]. NI Reis SR, Sampaio ALF, das Gracas Muller Henriques M, Gandini M, Leal Azeredo E, Fernandes Kubelka C. An *in vitro* model for dengue virus infection that exhibits human monocyte infection, multiple cytokine production and dexamethasone immunomodulation. Mem. Inst. Oswaldo Cruz. 2007; 102(8):983-90.

[18]. Lin CF, Chiu SC, Hsiao YL, Wan SW, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Lin YS. Expression of cytokine, chemokines, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. J. Immunol. 2005; 175:395-403.

[19]. Bosch I, Xhaja K, Estevez L, Raines G, Melichar H, Warke RV, Fournier MV, Ennis FA, Rothman AL. Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. J. Virol. 2002; 76:5588-97.

[20]. Bio-Rad. Bio-Plex Pro™ assays: cytokine, chemokine, and growth factors, instruction manual, revision A. Bio-Rad Laboratories, Inc., Hercules, CA; 2008.

[21]. Kleiner G, Marcuzzi A, Zanin V, Monasta L, Zauli G. Cytokine levels in the serum of healthy subjects. Mediators of Inflammation. 2013; dx.doi.org/10.1155/2013/434010.

[22]. Yeo AS, Azhar NA, Yeow, W, Talbot CC Jr, Khan MA, Shankar EM, Rathakrishnan A, Azizan A, Wang SM, Lee SK, Fong MY, Manikam R, Devi Sekaran S. Lack of clinical manifestations in asymptomatic dengue infection is attributed to broad down-regulation and selective up-regulation of host defense response genes. PLoS One. 2014; doi:10.1371/journal.pone.0092240.

[23]. Wuyts A, Struyf S, Proost P, van Damme J. Chemokines. In: Theze J, editor. The Cytokine Network and Immune Functions. New York: Oxford University Press; 1999. p. 125-45.

[24]. Halstead SB. Pathophysiology. In: Halstead SB, editor. Dengue. London: Imperial College Press; 2010. p. 285-326.

Table 3.1: Cytokine and chemokine target analytes of commercially available 27-plex MIA.
Analytes in bold denote those chosen for subsequent 5-plex custom MIA.

27-plex MIA cytokine kit (target analytes):			
IL-1β	IL-8	Eotaxin	MIP-1α
IL-1ra	IL-9	Basic FGF	MIP-1β
IL-2	**IL-10**	G-CSF	PDGF-BB
IL-4	IL-12(p70)	**GM-CSF**	RANTES
IL-5	IL-13	**IFN-γ**	TNF-α
IL-6	IL-15	**IP-10**	VEGF
IL-7	IL-17	**MCP-1**	

Table 3.2: Average concentrations (pg/mL) of the 5 analytes chosen for inclusion in 5-plex MIA in two different sets of serum samples. The table below lists the average observed concentrations (pg/mL) of each of the five chosen analytes for inclusion in a multiplexed DENV MIA (IL-10, GM-CSF, IFN-γ, IP-10, and MCP-1) for the two sample sets included for study. The first sample set (top) included diagnostics via serology only, with samples obtained from the BPHL-Tampa archive. The second sample set included both molecular and serological diagnostic methods from BPHL-Tampa archived samples in addition to samples obtained from a serosurvey in an outbreak setting (Martin County, FL). Note that the inclusion of DENV qRT-PCR+ samples led to much higher detection of concentrations of all five analytes via MIA. Here, listed values are average concentrations of each sample group rather than individual samples Range of values are included parenthetically, 0 values represent those below limits of detection. (* denotes values in range that were above detection limits, n=5 for [IP-10] S1 DENV+, n=1 for [MCP-1] DENV PCR+, <LOD-below limit of detection).

	Average [target analyte] pg/mL				
Sample subset	**[IL-10]**	**[GM-CSF]**	**[IFN-γ]**	**[IP-10]**	**[MCP-1]**
S1 DENV+	**7.53**(0-40.3)	<LOD	**43.05**(0-115.09)	**469.18**(0-1095.27*)	**48.49**(0-231.82)
S1 FLAVI+	**0.04**(0-0.15)	<LOD	**43.51**(30.25-66.05)	**395.44**(309.7-463.63)	**57.80**(24.98-143.37)
S2 DENV+	**0.97**(0-6.43)	<LOD	**22.47**(2.38-47.46)	**2039.08**(327.61-8679.03)	**48.86**(15.87-94.88)
S2 FLAVI+	**0.01**(0-0.04)	<LOD	**44.26**(24.96-66.5)	**1168.93**(252.57-2532.72)	**35.21**(11.97-58.29)
Neg (DOH)	**0.09**(0-0.18)	<LOD	**42.06**(0-136.38)	**2779.55**(0-6685.01)	**47.58**(5.01-152.75)
DENV PCR+	**86.75**(1.28-330.17)	**7.12**(0-96.7)	**241.08**(0-1879.6)	**19114.99**(40.81-56756.27)	**505.91**(43.12-1500.98*)
DENV+ IgG only	**2.10**(1.16-3.04)	**0.00**	**90.82**(0-181.64)	**6818.85**(1757.9-11879.79)	**189.11**(63.13-315.09)
DENV neg (DOH)	**4.65**(0.32-8.97)	**0.00**	**12.82**(0-25.64)	**13995.25**(1158.47-26832.03)	**165.30**(93.02-237.58)
DENV neg (serosurvey)	**0.47**(0-0.76)	**11.52**(0-21.41)	**52.41**(0-95.61)	**769.62**(475.98-1176.56)	**67.77**(42.06-84.86)

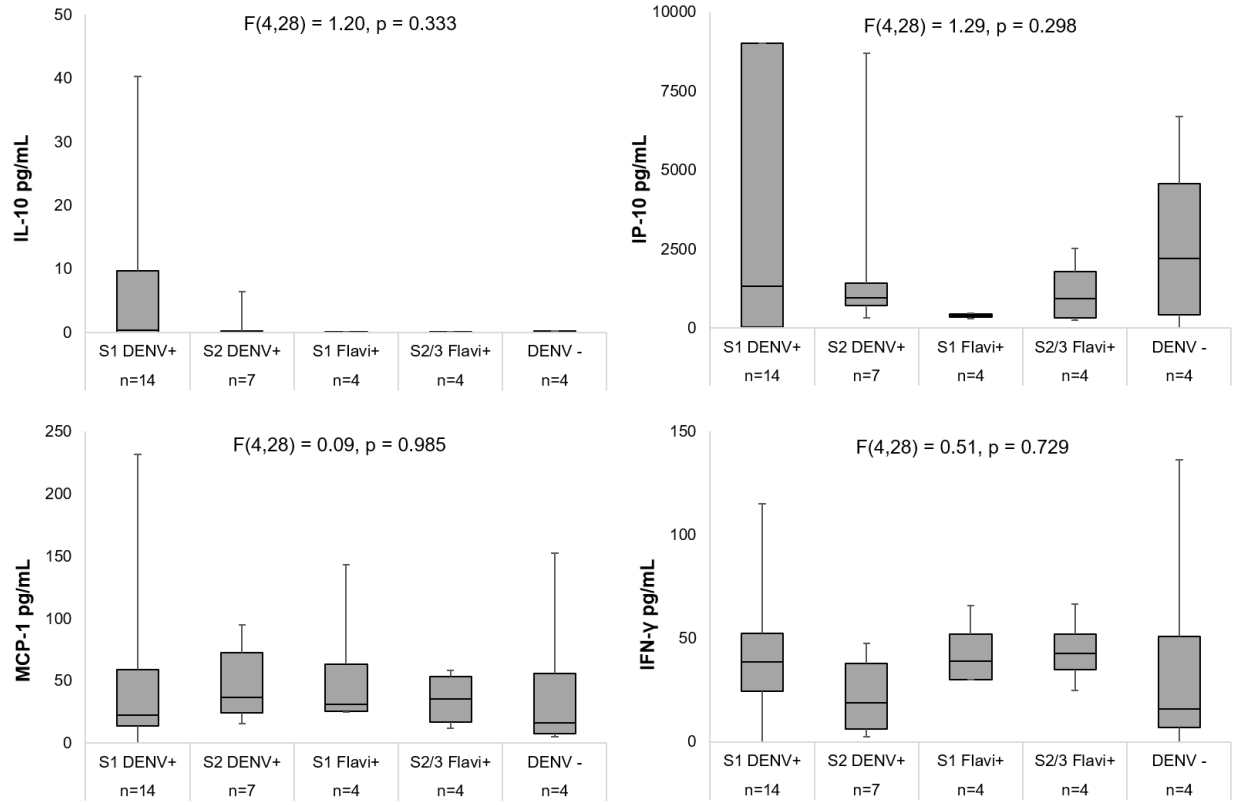

Figure 1a-d

Fig. 3.1a-1d: Production of IL-10, IFN-γ, IP-10, and MCP-1 in serum samples as determined by 27-plex MIA as illustrated by box-plot. Figures 1a-1d contain boxplots comparing DENV positive (acute and convalescent), WNV/Flavivirus positive (acute and convalescent) and DENV negative sera analyte concentrations as determined via 27-plex MIA. Four analytes are shown: IL-10, IFN-γ, IP-10 and MCP-1. Upper and lower bars represent the maximum and minimum concentration values obtained for each analyte within each subgroup, respectively. Top and bottom portions of the boxes mark the second and third quartiles and together represent the interquartile range (IQR). Lines within the boxes represent median concentration values. One-way ANOVA was used to assess differences between groups, with results shown at the top of each figure. A fifth analyte, GM-CSF is not shown in this series of figures as corresponding assay values were below the limits of detection for all sera sample subsets. Note that no statistically significant differences were observed between groups for any of the above 4 analytes.

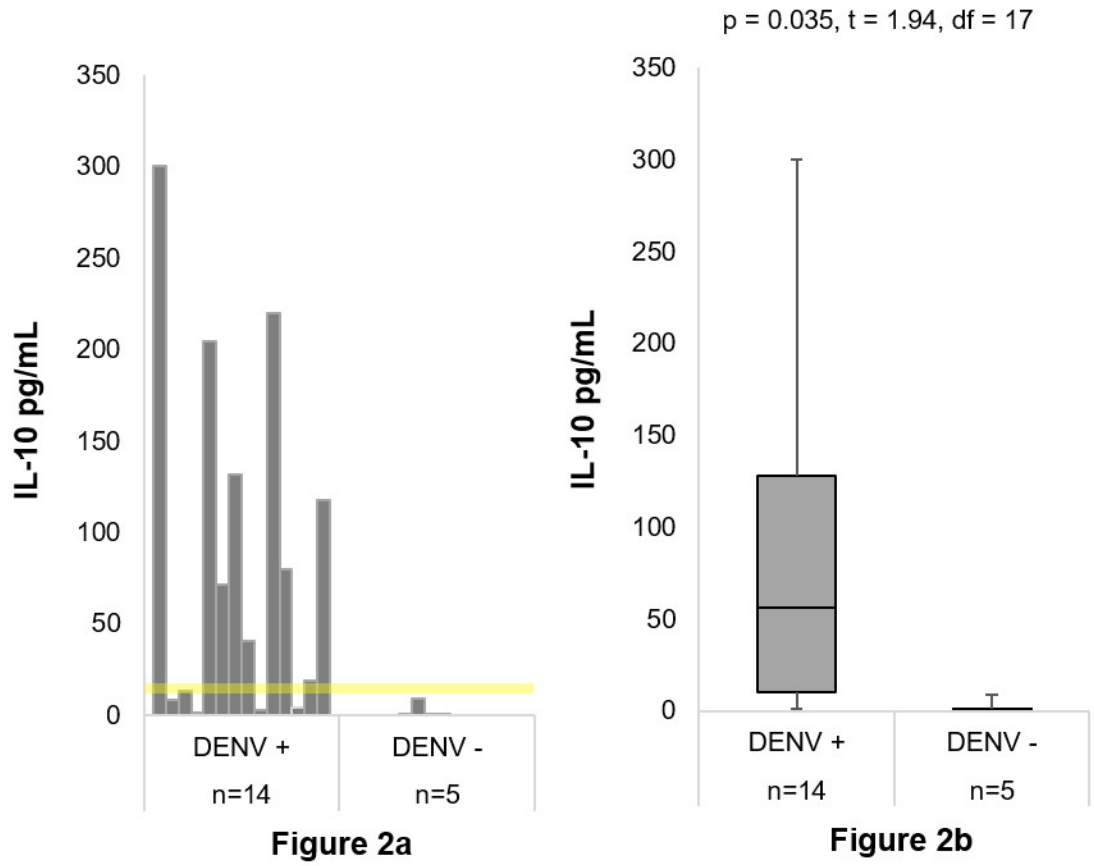

Fig. 3.2a-2b: IL-10 production (pg/mL) in serum samples as determined by 5-plex MIA. Figure 2a illustrates the observed average concentrations (pg/mL) of IL-10 in individual specimens as determined by MIA for DENV PCR+ and DENV- samples. Figure 2b shows boxplots of IL-10 concentrations (pg/mL), by DENV diagnostic group [DENV+, (n=14) and DENV- (n=5)]. Upper and lower bars represent the maximum and minimum concentration values obtained for IL-10 within each subgroup, respectively. Top and bottom portions of the boxes mark the second and third quartiles and together represent the interquartile range (IQR). Results of t-tests are also shown in Fig. 2b Here, the IQR of DENV PCR+ samples lies entirely above that of DENV- samples, and the difference between groups was statistically significant (p=0.035). Furthermore, 8 of the 14 concentration values of DENV PCR+ sera were markedly higher than those in the DENV negative group. Note that the shaded area represents the range of concentrations obtained for each analyte when also measured by MIA in healthy individuals within a separate study where most values obtained from DENV PCR+ sera were found to significantly exceed this range and all DENV- values were found to be below this range[21].

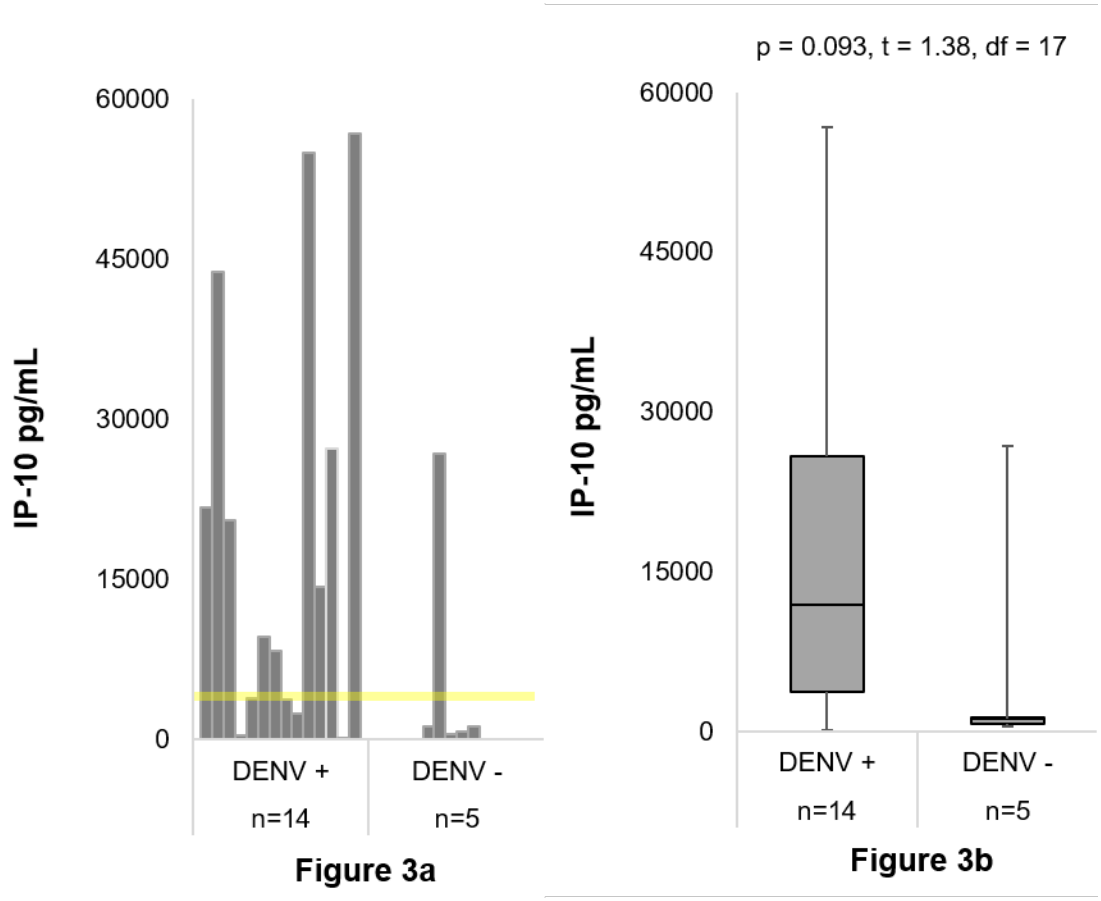

Fig. 3.3a-3b: IP-10 production (pg/mL) in serum samples as determined by 5-plex MIA.
Figure 3a illustrates the observed average concentrations (pg/mL) of IP-10 in individual specimens as determined by MIA for DENV PCR+ and DENV- samples. Figure 3b contains boxplots detailing IP-10 concentrations (pg/mL) by DENV diagnostic group [DENV+, (n=14) and DENV- (n=5)]. Upper and lower bars represent the maximum and minimum concentration values obtained for IP-10 within each subgroup, respectively. Results of t-tests are also shown in Fig. 3b. While statistically significant differences between groups were found to be borderline (p=0.093), note that the IQR of DENV PCR+ samples lies entirely above that of DENV- samples. Additionally, the maximum concentration values between groups was nearly two-fold higher for DENV PCR+ sera (56756.27pg/mL compared to 26832.03pg/mL). Note that the shaded area represents the range of concentrations obtained for each analyte when also measured by MIA in healthy individuals within a separate study[21].

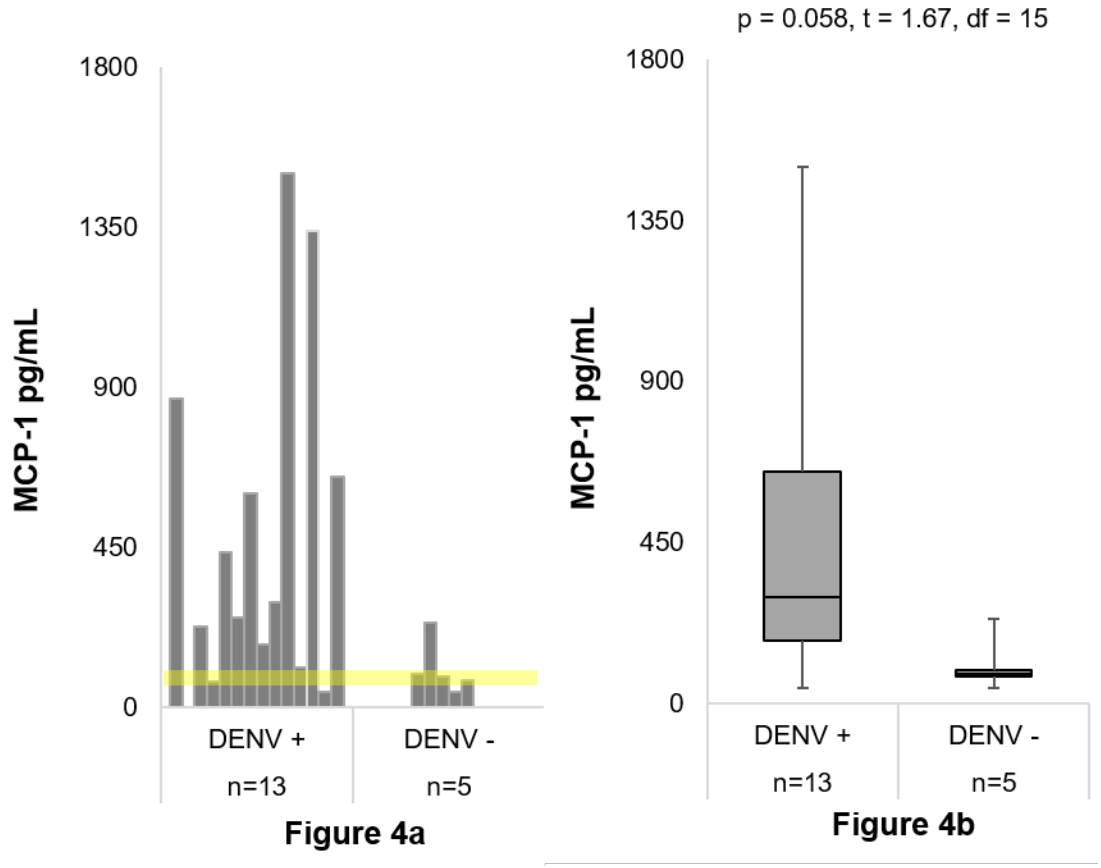

Fig. 3.4a-4b: MCP-1 production (pg/mL) in serum samples as determined by 5-plex MIA.
Figure 4a illustrates the observed average concentrations (pg/mL) of MCP-1 in individual specimens as determined by MIA for DENV PCR+ and DENV- samples. Figure 4b contains boxplots detailing MCP-1 concentrations (pg/mL) by DENV diagnostic group [DENV+, (n=13) and DENV- (n=5)]. Results of t-tests are also shown in Fig. 4b. While statistical differences between groups were found to be borderline (p=0.058) being slightly above the test threshold of p=0.05, observe that the IQR of DENV PCR+ samples lies entirely above that of DENV- samples. Also, the maximum concentration values between groups was much higher in the DENV+ group than that of DENV- samples (1500.98pg/mL compared to 237.58pg/mL). This also does not include a DENV PCR+ sample that was not included in analysis due to the fact that its value was beyond that of the detection limits of the assay. Note that the shaded area represents the range of concentrations obtained for each analyte when also measured by MIA in healthy individuals within a separate study[21].

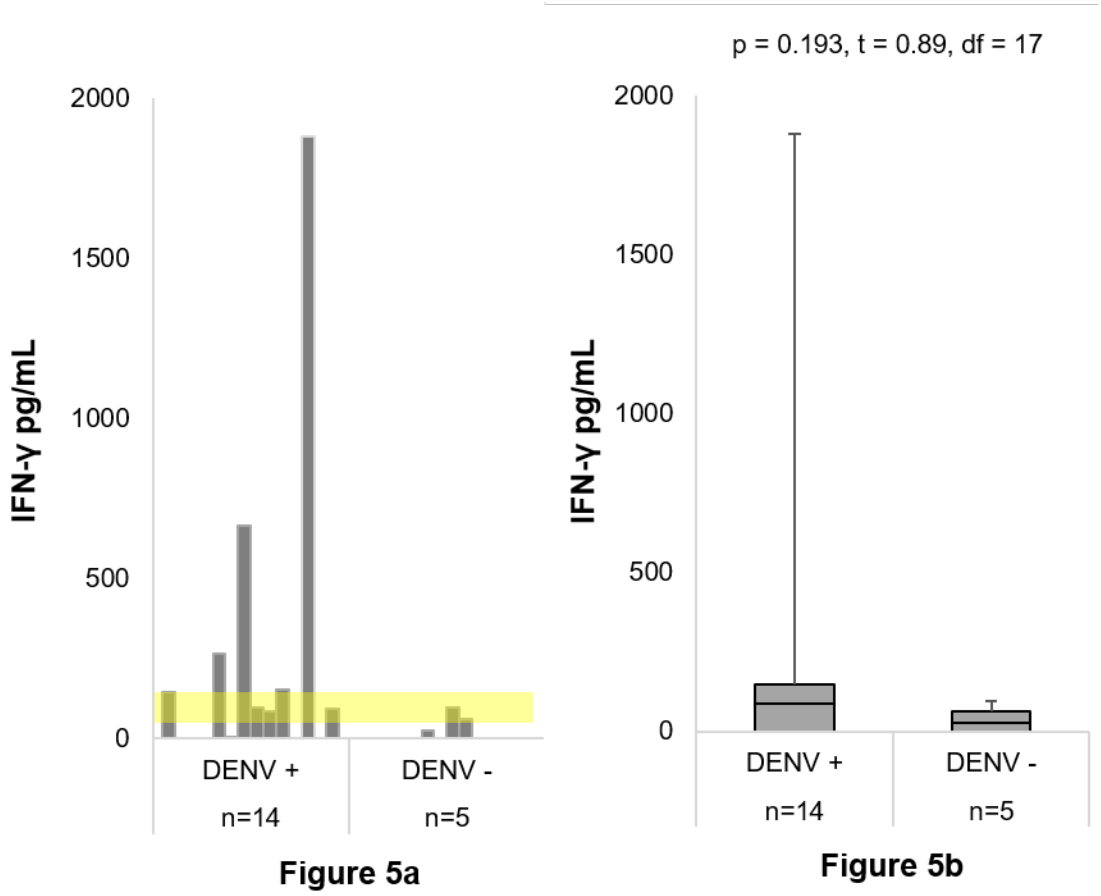

Fig. 3.5a-5b: IFN-γ production (pg/mL) in serum samples as determined by 5-plex MIA.
Figure 5a illustrates the observed average concentrations (pg/mL) of IFN-γ in individual specimens as determined by MIA for DENV PCR+ and DENV- samples. Figure 5b contains boxplots detailing IFN-γ concentrations (pg/mL) by DENV diagnostic group [DENV+, (n=14) and DENV- (n=5)]. Upper and lower bars represent the maximum and minimum concentration values obtained for IFN-γ within each subgroup, respectively. Top and bottom portions of the boxes mark the second and third quartiles and together represent the interquartile range (IQR). Results of t-tests are also shown in Fig. 5b. While statistically significant differences were not found between groups (p=0.193), and the IQRs appear similar, please observe that 5 of the 14 DENV PCR+ samples detected IFN-γ concentrations at least as twice a high or higher than the maximum concentration value of DENV- samples (95.61pg/mL) including one dramatically higher (1879.6pg/mL). Note that the shaded area represents the range of concentrations obtained for each analyte when also measured by MIA in healthy individuals within a separate study[21].

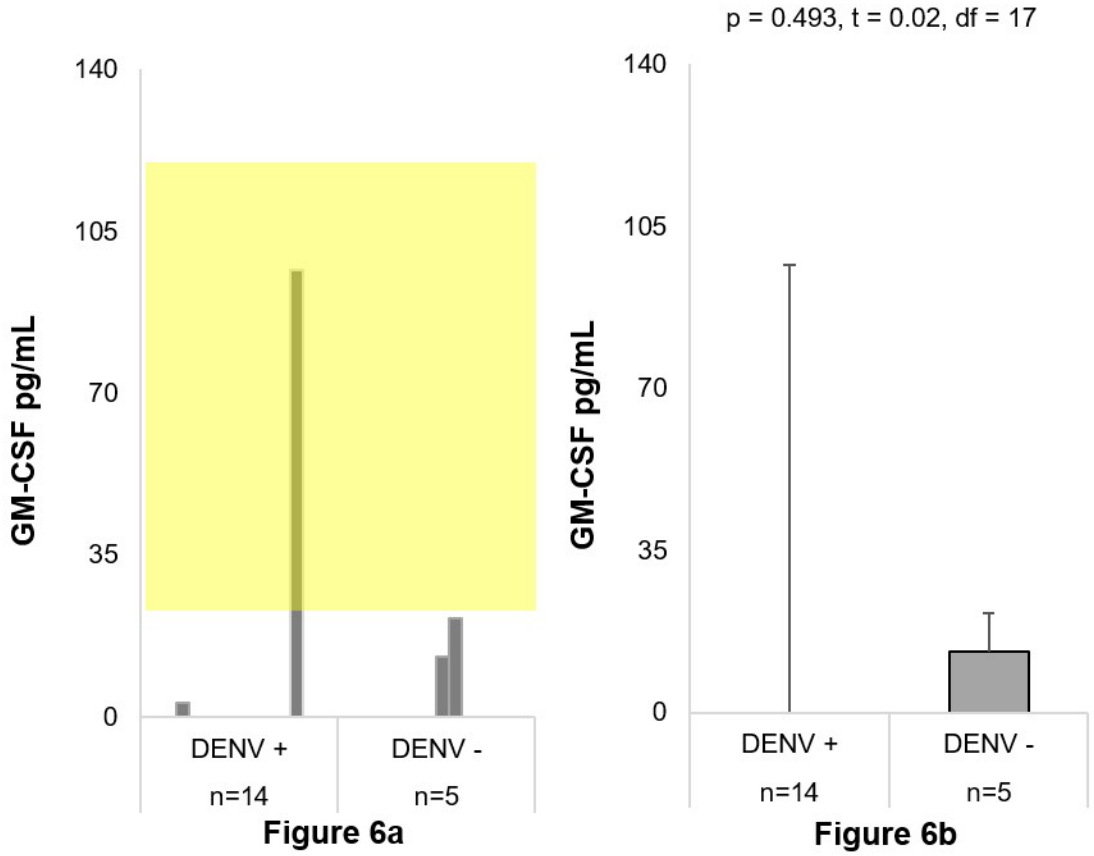

Fig. 3.6a-6b: GM-CSF production (pg/mL) in serum samples as determined by 5-plex MIA. Figure 6a illustrates the observed average concentrations (pg/mL) of GM-CSF in individual specimens as determined by MIA for DENV PCR+ and DENV- samples. Figure 6b contains boxplots detailing GM-CSF concentrations (pg/mL) by DENV diagnostic group [DENV+, (n=14) and DENV- (n=5)]. Upper and lower bars represent the maximum and minimum concentration values obtained for GM-CSF within each subgroup, respectively. Top and bottom portions of the boxes mark the second and third quartiles and together represent the interquartile range (IQR). Results of t-tests are also shown in Fig. 6b. Statistically significant differences were not observed between groups (p=0.493). Also, observe the maximum concentration value in the DENV PCR+ group (96.7pg/mL) which may represent a potential but significant outlier. Note that the shaded area represents the range of concentrations obtained for each analyte when also measured by MIA in healthy individuals within a separate study and that all values obtained here fell within or below this range[21] .

CHAPTER 4: DENGUE VIRUS NS1 PROTEIN AS A DIAGNOSTIC MARKER: COMMERCIALLY AVAILABLE ELISA AND COMPARISON TO qRT-PCR AND SEROLOGICAL DIAGNOSTIC ASSAYS CURRENTLY USED BY THE STATE OF FLORIDA. AMBROSE, J.H., S.D. SEKARAN, AND A. AZIZAN. 2017. J. TROP. MEDICINE. HTTPS://DOI.ORG/10.1155/2017/8072491

This manuscript was published in the Journal of Tropical Medicine and was reprinted under Open Access policies of said journal. It appears here in the form it was submitted, according to Journal of Tropical Medicine formatting with the exception of minor changes requested by the doctoral committee and appropriate headings for Sections, Tables, and Figures reflecting internal formatting of this document based on Chapter. (*Also note that there was a corrigendum submitted for this article where the caption for Table 4.1 should read 'of less than' instead of '>' and this correction is reflected here).*

Dengue virus NS1 protein as a diagnostic marker: commercially available ELISA and comparison to qRT-PCR and serological diagnostic assays currently used by the state of Florida

Jason H. Ambrose[1,2], Shamala Devi Sekaran[3], and Azliyati Azizan[1,4]

[1]Global Health Department, College of Public Health, University of South Florida, 12901 Bruce B Downs Blvd., Tampa, FL, 33612, USA (ajason@health.usf.edu; aazizan@health.usf.edu)

[2]Bureau of Public Health Laboratories, Florida Department of Health, 3602 Spectrum Blvd. Tampa, FL, 33612, USA

[3]Department of Medical Microbiology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, Malaysia (shamalamy@yahoo.com; shamala@um.edu.my; shamala@ummc.edu.my)

[4]Nazarbayev University School of Medicine (NUSOM), 53 Kabanbay Batyr Ave., Astana, 010000, Republic of Kazakhstan (azliyati.azizan@nu.edu.kz)

Corresponding author:

Azliyati Azizan, PhD, MS

Nazarbayev University School of Medicine (NUSOM)

53 Kabanbay Batyr Ave.

Astana, Republic of Kazakhstan, 010000

Email: azliyati.azizan@nu.edu.kz

4.1. Abstract

Background: The proper management of patients infected with dengue virus requires early detection. Here, real-time molecular assays have proven useful but have limitations, whereas ELISAs that detect antibodies are still favored but results obtained too late to be of clinical value. The production of DENV NS1 peaks early during infection and its detection can combine the advantages of both diagnostic approaches.

Methods: This study compared assays currently used for detecting DENV infection at the Florida Department of Health including anti-DENV IgM and IgG ELISAs as well as qRT-PCR, against a commercially available DENV NS1 ELISA. These comparisons were made among a group of 21 human sera.

Results: Nine of 14 (64.3%) DENV qRT-PCR+ samples were also DENV NS1+. Interestingly, the 5 NS1- samples that were qRT-PCR+ were additionally IgM- and IgG+ suggesting a non-primary infection. Compared to qRT-PCR, the NS1 assay had a sensitivity of 64.3% and specificity of 100%.

Conclusions: The NS1 ELISA performed as expected in known DENV qRT-PCR+ samples, however negative NS1 results for qRT-PCR+, IgG+ sera seemingly reduced the usefulness of the NS1 ELISA for non-primary cases. We therefore conclude that diagnosis obtained via DENV NS1 ELISA deserves further investigation.

Key words: dengue; DENV; NS1; ELISA; diagnostics

4.2. Introduction

Infections caused by dengue virus continue to constitute a worldwide threat to the public, both in human and economic costs. In 2017, dengue virus remains the cause of one the most globally significant

arthropod-borne (arbo-) viral illnesses. According to WHO, there is currently an at risk global population of 3.9 billion where an estimated 390 million infections occur annually. Around 96 million infected persons seek clinical attention but the majority of cases go unreported. Approximately 500,000 of clinical patients will progress to severe illness and require hospitalization with fatalities arising in 2.5%[1,2]. Additionally, after a 75-year absence, local transmission of DENV was documented in Florida, USA. During the time period between 2009-12, 103 autochthonous cases were documented with the majority of those cases (27 in 2009 and 63 in 2010) associated with an outbreak of DENV serotype 1 (DEN1) in Key West (Monroe County). However, epidemiologically unrelated, locally acquired cases were also documented in Broward, Hillsborough, Miami-Dade, Palm Beach, Osceola, Martin, and Seminole counties through 2012. A second outbreak of DEN1 occurred in Martin County during 2013[3-5]. Two introductions of dengue are thought to have occurred in Martin County, the first near Port Salerno in 2011 and the second near Jensen Beach; the latter introduction responsible for the outbreak. In 2016, a case of DEN4 (Cone M, *personal comm*.) was locally-acquired in Key West[6] and dengue appeared again locally in Miami-Dade during Zika virus outbreak investigations[7].

Dengue is caused by one of four different serotypes of small RNA viruses in the family *Flaviviridae*, DENV1-4. The 5' and 3' ends of the DENV genome contain untranslated regions (UTRs) and the open reading frame first encodes the three structural proteins, C, prM/M, and env, followed by 7 non-structural (NS) proteins, including the NS1 protein. The genome is translated as a single polyprotein that is processed and modified post-translationally [8]. The NS1 protein itself is secreted from infected cells and is found in serum at detectable levels that overlap with peak viremia (and RNA detection) in addition to the onset of detectable antibody levels of IgM in acute primary cases and IgG in acute non-primary cases[9]. It has been found that elevated levels of serum NS1 directly indicates increased viral burden and further establishes the positive correlation between viremia and NS1 profiles[10,11]. NS1 is a generally conserved protein among flaviviruses but has been found to contain both cross-reactive and serotype-specific epitopes among dengue viruses; these are important factors when considering development of immunoassays[12-14]. For these reasons NS1 is considered as having diagnostic value as a viral marker of infection. The protein is found both intracellularly as well as in a soluble form (sNS1) secreted from infected host cells but its function remains enigmatic. The immature form of NS1 is that of

a monomer that is variably glycosylated but readily forms heat-labile homodimers usually associated with the surface of infected cells[8,14]. From there, the major oligomeric form of sNS1 is thought to be a hexamer of around 300kD. The hexamer consists of 3 dimeric subunits that are non-covalently bound and are less stable than NS1 dimers[15,16].

Dengue is a problematic disease to manage at the clinical level, in large part due to late manifestations of severe illness in some patients[17]. In the past, techniques including virus isolation and serological assays such as ELISA and plaque-reduction neutralization assays (PRNT) typically yielded results after clinical resolution (or development of severe illness), leading to diagnosis with no benefit to the patient. However, the capabilities of the laboratory have advanced to the level of obtaining same day results in acutely infected patients with the advent of rapid techniques that include molecular diagnostic assays such as real-time qRT-PCR[9]. As alluded to above, the NS1 protein of DENV is also a useful early viral marker of infection and an ELISA (Pan-Bio® Dengue Early ELISA #E-DEN02P) that detects it is currently available from Inverness Medical (now Alere Inc.) among other immunoassay manufacturers. These DENV NS1 immunoassays may represent the new paradigm for DENV diagnosis in many parts of the world, in part, by serving to combine the pros of both traditional serological assays with those of modern molecular assays. These include early diagnosis commensurate with the capabilities of molecular assays coupled with decreased costs in both equipment and reagents along with reducing the technical prowess and stringency required for performing clinical molecular assays. This study attempted to ascertain the potential value of the DENV NS1 ELISA in diagnosing dengue in the US state of Florida.

4.3. Materials and Methods

4.3.1. Ethics Statement

The removal of identifiers in these previously collected serum samples resulted in the determination of this study as not meeting the definition of human research activities and thus IRB exempt under US 45 CFR 46.101(4). This ruling was determined by the University of South Florida IRB.

4.3.2. Sample Selection

A series of 21 serum samples previously assayed at the Florida Department of Health (FLDOH)-Bureau of Public Health Laboratories (BPHL)-Tampa for DENV by qRT-PCR and either, anti-DENV IgM and IgG ELISA in concert (20/21) or IgM only (1/21), were subsequently subjected to DENV NS1 detection by ELISA. Corresponding DENV serotypes of positive samples were also obtained via qRT-PCR. Eight of 14 qRT-PCR+ samples were positive for DEN1, five were DEN4+, and one was DEN2+. No samples that were identified as DEN3+ were included in this study. Dengue qRT-PCR- samples belonged to either of the following diagnostic categories: anti-DENV IgG+ (n=2) or samples negative for all routine DENV diagnostic assays. The latter samples were obtained from either the clinical archive (n=2) or from a serosurvey (n=3) conducted in Martin County, Florida during the course of an outbreak of DEN1. The DENV IgG and IgM ELISAs were adapted from protocols provided by CDC-Arboviral Diseases Branch (Ft. Collins, CO) and TaqMan-based DENV serotype-specific qRT-PCR was performed using an FDA-approved protocol provided by CDC-Dengue Branch (San Juan, Puerto Rico).

4.3.3. DENV NS1 ELISA

The PanBio® dengue early ELISA (Inverness Medical, Sinnamon Park, QLD, Australia #E-DEN02P) was used to determine the presence of DENV NS1 in individual serum samples. Each sample was ran in duplicate, according to manufacturer's instructions. The assay was performed according to manufacturer's instructions with the following changes specific to our study. Samples, positive controls, and negative controls were added to wells in duplicate and calibrators were added in quadruplicate, all at 100µL. The ELISA sample plates were read at 450nm with a reference filter of 620nm. Each sample optical density (OD) value (absorbance) was averaged between duplicate wells then divided by the cut-off value to obtain index values. Values <0.9 were ruled as negative, between 0.9 and 1.1 as equivocal, and values above 1.1 positive for DENV NS1 detection. The results of this assay were then compared to those of DENV virus RNA detection via qRT-PCR as well as anti-DENV IgM and IgG ELISAs previously performed at the FLDOH-BPHL-Tampa.

4.4. Results

In our study, the DENV NS1 ELISA (Tables 4.1 and 4.2) found 9 of 14 sera to be positive for DENV that were also qRT-PCR+ and 0 of 2 that were previously positive by IgG only (9/16 total DENV+ samples). Five samples that were DENV- negative in all 3 comparison assays, including 2 clinical samples and 3 from the Martin County serosurvey, were also negative via DENV NS1 ELISA. Each of the DENV qRT-PCR+ samples that were found to be NS1 ELISA- were also negative for IgM (5/14). Interestingly, however, these 5 qRT-PCR+ samples were positive for IgG (and IgM-) by ELISA, suggesting non-primary infection. Additionally, each of the 3 dengue serotypes represented in this study were found within this subgroup (3 of 5 samples were DEN1, 1=DEN2, and 1=DEN4). On the other hand, one sample that was NS1+ presented with the same profile (*i.e.* qRT-PCR+, IgM-, and IgG+). All seven qRT-PCR- samples were also negative for DENV NS1. Please take note that the desired direct comparison between assays was that made between those capable of early detection (NS1 ELISA vs. qRT-PCR). Therefore, while both assays failed to detect dengue in 2 samples that were IgG+ only, the 'true negative' sample number of (n=7) was left to stand for calculations so as not to skew the NPV artificially in favor of qRT-PCR. In all and when compared to the results of qRT-PCR, the NS1 assay was found to have a sensitivity of 64.3% and specificity of 100%.

4.5. Discussion

Like the results found here, previous reports of the investigational use of commercially available DENV NS1 immunoassays such as those in Brazil[18] and Malaysia[19] showed favorable results when compared against standard diagnostic methods. The former group used the Platelia™ Dengue NS1 Ag microplate EIA (Bio-Rad, Hercules, CA) and the latter the SD Bioline Dengue Duo (Standard Diagnostics, Yongin-si, Rep. Korea). These groups obtained results with sensitivities of 95.9% and 65.41% and specificities of 81.1% and 98.75%, respectively (whereby for the SD Bioline assay, a multiplex assay, only NS1 was considered). In 2010, the Platelia™ assay was approved for the screening of 80,000 Puerto Rican blood donors. At the time of FDA approval for the particular study, the test was already in use in

approximately 40 countries around the world[20]. The USNIH notes on www.clinicaltrials.gov that the study has been completed but official results have yet to be reported[21]. In Lima MdaR *et al*.[22], two of the previously mentioned assays, the PanBio® ELISA and the Platelia™ EIA, were compared against another immunoassay, the Dengue NS1 STRIP (Bio-Rad, Hercules, CA). The STRIP is an immunochromatographic test similar to the previously mentioned SD Bioline Dengue Duo. While all obtained specificities near 100%, here they found the STRIP assay to have the highest sensitivity (89.6%), followed by the Platelia™ EIA at 83.6%, and the Pan-Bio® ELISA at 72.3%. This group also reported that the assays were less sensitive in detecting DEN3 cases and that the Platelia™ assay detected primary cases at a statistically significant higher percentage than non-primary cases.
It should also be noted that concerns about the sensitivity of the Platelia™ EIA arose in a study in Aracaju, Brazil where 58 of 119 NS1 negative samples were instead found later to be DEN4+ by confirmatory tests, and their reasoning pointed to an issue with the detection of non-primary cases[23]. As mentioned above, this was also seemingly evident in our study where all 5 NS1 ELISA- samples known to be qRT-PCR+ were also IgG+ in ELISA. However, a subsequent report published after obtaining the results reported here, indicated that this drawback can be alleviated by pre-heating samples at 100°C for 5m[24]. This would indicate that the assay may require dissociation of antigen-antibody complexes and/or preferentially detects monomeric NS1 over its dimeric form. The former seems very likely as, in non-primary infections, NS1 bound by IgG antibodies produced during the early phases would reduce the pool of free and detectable serum levels of this protein. On the other hand, their data suggested that with heating, the assay is preferentially detecting NS1 monomers in both types of dengue infection[14]. It would be important to empirically determine that the heating step reported above is producing dissociation of antigen-antibody complexes and/or dissociation of free dimeric NS1 into constituent monomers and that this step is essential for increasing sensitivity in both types of infection.

There also remains the concern that no single assay included in this study was alone sufficient for diagnosis. This was evident where 2 out of 7 qRT-PCR- samples were found to be DENV+ only via IgG detection. This in turn affected the comparison between the test under question (DENV NS1 ELISA) and the gold-standard used here for early detection (qRT-PCR). Wang and Sekaran[19] abrogated this assay-related issue to a large extent through the use of a ‘one-stop’ rapid test able to detect not only NS1 but

also IgM and/or IgG. This in turn increased the sensitivity of their combined assay and identified, concurrently, more positive individuals. This and other multifaceted approaches to DENV diagnostics seems to be the proper direction moving forward and we encourage further investigation.

Regarding our study, we accept that larger scale studies typically include greater numbers of negative samples when characterizing new assays. Here, though, both the limited number of reagents as well as DENV+ samples available for study compelled us to approach the study from the opposite direction. Additionally, as part of a larger study, these samples were also subject to analyses, such as immunological profiling[25] and experimental DENV NS1 detection, further supporting the approach used here. Despite the small sample size included, we nevertheless conclude that assays detecting DENV NS1 should eventually be incorporated within the algorithms of laboratories performing dengue diagnostics, including BPHL-Tampa. We also propose that they are investigated for further utility, especially in conjunction with not only other potential diagnostic markers, but also those of prognostic value, in order to better inform the clinic in identifying and properly managing patients infected with dengue.

4.6. References

[1]. World Health Organization (WHO). Dengue and severe dengue. 2015. http://www.who.int/mediacentre/factsheets/fs117/en/. Accessed 15 Aug 2015.

[2]. Gullard A. Burden of dengue fever is higher than previously thought. BMJ. 2013; 347:f6280; doi:10.1136/bmj.f6280.

[3]. Florida Health. Dengue Fever- dengue occurrence in Florida. 2015. http://www.floridahealth.gov/diseases-and-conditions/dengue/. Accessed 18 August 2015.

[4]. Munoz-Jordan JL, GA Santiago, H Margolis, L Stark. Genetic relatedness of dengue viruses in Key West, Florida, USA, 2009-2012. EID. 2013; 19(4):652-4.

[5]. Anez G, DAR Heisey, LM Espina, SL Stramer, M Rios. Phylogenetic analysis of dengue virus types 1 and 4 circulating in Puerto Rico and Key West, Florida, during 2010 epidemics. Am. J. Trop. Med. Hyg. 2012; 87(3):548-53.

[6]. Florida Health. Health officials issue mosquito-borne illness advisory. 2016. http://www.floridahealth.gov/diseases-and-conditions/mosquito-borne-diseases/_documents/2016/monroe-dengue-5-31-16.pdf. Accessed 3 October 2016.

[7]. Miami Herald. First Zika, now dengue. New case pops up in Miami area. 2016. http://www.miamiherald.com/news/health-care/article104606196.html. Accessed 3 October 2016.

[8]. Beasley DWC, ADT Barrett. The infectious agent. In: Halstead SB, editor. Dengue. London: Imperial College Press; 2010. p. 29-73.

[9]. World Health Organization (WHO). Dengue: guidelines for diagnosis, treatment, prevention and control- new edition. Geneva: WHO Press; 2009.

[10]. Avirutnan P, N Punyadee, S Noisakran, C Komoltri, S Thiemmeca, K Auethavornanan, A Jairungsri, R Kanlaya, N Tangthawornchaikul, C Puttikhunt, SN Pattanakitsakul, PT Yenchitsomanus, J Mongkolsapaya, W Kasinrerk, N Sittisombut, M Husmann, M Blettner, S Vasanawathana, S Bhakdi, P Malasit. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis. 2006; 93(8):1078-88.

[11]. Libraty DH, PR Young, D Pickering, TP Endy, S Kalayanarooj, S Green, DW Vaughn, A Nisalak, FA Ennis, AL Rothman. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J. Infect. Dis. 2002; 186(8):1165-8.

[12]. Ding XX, DM Hu, Y Chen, B Di, J Jin, YX Pan, LW Qiu, YD Wang, K Wen, M Wang, XY Che. Full serotype- and group-specific NS1 capture ELISA for rapid differential diagnosis of dengue infection. Clin. Vacc. Immunol. 2011; doi:10.1128/CVI.00462-10.

[13]. Falconar AKI. Monoclonal antibodies that bind to common epitopes on the dengue virus type 2 nonstructural-1 and envelope glycoproteins display weak neutralizing activity and differentiated responses to virulent strains: implications for pathogenesis and vaccines. Clin. Vaccine Immunol. 2008; 15(3):549-61.

[14]. Falconar AKI, PR Young. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1. J. Gen. Virol. 1991; 72:961-5.

[15]. Gutsche I, F Coulibaly, JE Voss, J Salmon, J d'Alayer, M Ermonval, E Larquet, P Charneau, T Krey, F Mégret, E Guittet, FA Rey, M Flamand. Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. PNAS. 2011; 108(19):8003-8.

[16]. Flamand M, F Megret, M Mathieu, J Lepault, FA Rey, V Deubel. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J. Virol. 1999; 73(7):6104-6110.

[17]. Halstead SB. Dengue. Lancet. 2007; 370:1644-52.

[18]. Castro-Jorge LA, PRL Machado, CA Favero, MC Borges, LMR Passos, RM de Oliveira, BAL Fonseca. Clinical evaluation of the NS1 antigen-capture ELISA for early diagnosis of dengue virus infection in Brazil. J. Med. Virol. 2010; 82:1400-1405.

[19]. Wang SM, S Devi Sekaran. Evaluation of a commercial SD dengue virus NS1 antigen capture enzyme-linked immunosorbent assay kit for early diagnosis of dengue virus infection. J. Clin. Microbiol. 2010; 48(8):2793-97.

[20]. Bio-Rad. Bio-Rad receives notification from the FDA that it may proceed with an investigational new drug study for its dengue NS1 Ag microplate assay. 2010. http://www.bio-rad.com/en-us/corporate/newsroom/bio-rad-receives-notification-from-the-fda-that-it-may-proceed-with-an-investigational-new-drug-study-for-its-dengue-ns1-ag-microplate-assay?LocaleKey=. Accessed 3 October 2016.

[21]. US National Institutes of Health (NIH). Dengue virus NS1 antigen (Bio-Rad) clinical protocol. 2012. http://clinicaltrials.gov/ct2/show/NCT01226173. Accessed 3 October 2016.

[22]. Lima MdaR, RM Nogueira, HG Schatzmayer, FB dos Santos. Comparison of three commercially available dengue NS1 capture assays for acute diagnosis of dengue in Brazil. PLoS Negl. Trop. Dis. 2010; 4(7):e738; doi:10.1371/journak.pntd.0000738.

[23]. Sea VR, AC Cruz, RQ Gurgel, BT Nunes, EV Silva, SS Dolabella, RL dos Santos. Underreporting of dengue-4 in Brazil due to low sensitivity of the NS1 Ag test in routine control programs. PLoS One. 2013; 8(5):e64056; doi:10.1371/journal.pone.0064056.

[24]. Lima Mda R, RM Nogueira, AM Filippis, PC Nunes, CS Sousa, MH Silva, FB Santos. A simple heat dissociation method increases significantly the ELISA detection sensitivity of the nonstructural-1 glycoprotein in patients infected with DENV type-4. J. Virol. Methods. 2014; 204:105-8; doi:10.1016/j.viromet.2014.02.031.

[25]. Ambrose JH, LM Stark, JS Mateus, KA Fitzpatrick, A Azizan. Immunological profiles of human sera as determined by microsphere immunoassay (MIA) demonstrates that IL-10, IP-10, and MCP-1 levels are elevated in acute dengue infections. Microbiology and Virology. (Institute of Microbiology and Virology; Astana, Kazakhstan). 2016; 2(13):29-41. http://imv-kaz.kz/ru/2016-11-03-10-06-17.html

Table 5.1: DENV NS1 detection in selected serum samples as determined by ELISA and in comparison to clinical molecular (qRT-PCR) and serological (anti-DENV IgM and IgG) results. The table below details the results of DENV NS1 detection by ELISA against qRT-PCR, IgM, and IgG DENV assays for a group of serum samples selected for inclusion and based on the following criteria: 1- **denotes samples positive by qRT-PCR for DENV as determined by BPHL-Tampa** 2- **denotes samples positive for DENV by IgG detection only as determined by BPHL-Tampa** 3- **denotes samples that were DENV negative received by BPHL-Tampa for all DENV-specific assays** 4- **denotes samples that were collected from Martin County serosurvey and were found to be DENV negative by all DENV-specific assays**. Index values represent the mean of duplicate values obtained when reading samples at 450nm and taking calibrators into account. Negative samples had an index value of less than 0.9, those between 0.9-1.1 were equivocal, and those above 1.1 were positive for NS1 detection. Results are listed as either positive (+) or negative (neg) for each ELISA. Positive qRT-PCR results are reported either as neg. or positive by listing serotype and C_T value results. Samples that were qRT-PCR+ but NS1 neg. are highlighted within the table. Note that no single assay here was capable of diagnosing DENV infection alone.

sample	Index value	NS1 ELISA	qRT-PCR (C_T)	IgM ELISA	IgG ELISA
1-1	5.98	+	**DEN4** (20.40)	+	+
1-2	5.91	+	**DEN1** (14.19)	neg	neg
1-3	0.08	neg	**DEN1** (33.17)	neg	+
1-5	0.09	neg	**DEN1** (34.96)	neg	+
1-6	5.93	+	**DEN1** (26.06)	+	neg
1-7	5.93	+	**DEN1** (30.40)	+	neg
1-8	4.51	+	**DEN1** (22.32)	+	neg
1-9	5.38	+	**DEN1** (31.90)	+	neg
1-10	0.17	neg	**DEN1** (25.53)	neg	+
1-11	5.97	+	**DEN4** (29.79)	+	+
1-12	5.92	+	**DEN4** (20.74)	neg	+
1-13	0.21	neg	**DEN4** (19.69)	neg	+
1-14	0.25	neg	**DEN2** (25.13)	neg	+
1-15	6.00	+	**DEN4** (21.03)	+	+
2-3	0.43	neg	neg	neg	+
2-4	0.13	neg	neg	neg	+
3-1	0.05	neg	neg	neg	neg
3-2	0.09	neg	neg	neg	neg
4-1	0.05	neg	neg	neg	neg
4-2	0.06	neg	neg	neg	neg
4-3	0.06	neg	neg	neg	N/A

Table 5.2: Breakdown of DENV serological diagnostic status (any combination of DENV NS1, anti-DENV IgM, and/or –IgG) versus detection of DENV RNA via qRT-PCR. The table below details first the comparison of DENV NS1 detection via ELISA compared to results obtained for the respective sample set via qRT-PCR (n=21), set as a gold-standard. The table details a further breakdown of these results by including anti-DENV IgM and IgG status of the samples. Nine (9 out of 14) qRT-PCR+ samples were also DENV NS1+ (64.3%) and all 7 samples that were negative by qRT-PCR were also found to be negative for DENV NS1. Notably, all 5 DENV NS1- samples that were qRT-PCR+ were also anti-DENV IgM- and IgG+, while only 1 positive NS1 sample was found to have that same profile, indicating that non-primary infections may affect the sensitivity of the DENV NS1 ELISA. *Please note that 1 of the DENV qRT-PCR- samples was not assayed for DENV anti-IgG.

	DENV qRT-PCR results vs. ELISA	
DENV ELISA results	**DENV qRT-PCR+ (n=14)**	**DENV qRT-PCR- (n=7)**
DENV NS1+	9/14 **(64.3%)**	0/7 **(0%)**
DENV NS1-	5/14 **(35.7%)**	7/7 **(100%)**
DENV NS1+, IgM+	7/14 (50%)	0/7 (0%)
DENV NS1+, IgM-	2/14 (14.3%)	0/7 (0%)
DENV NS1-, IgM+	0/14 (0%)	0/7 (0%)
DENV NS1-, IgM-	**5/14 (35.7%)**	**7/7 (100%)**
DENV NS1+, IgG+	4/14 (28.6%)	0/6* (0%)
DENV NS1+, IgG-	5/14 (35.7%)	0/6* (0%)
DENV NS1-, IgG+	**5/14 (35.7%)**	2/6* (33.3%)
DENV NS1-, IgG-	0/14 (0%)	4/6* (66.7%)
DENV NS1+, IgM+, IgG+	3/14 (21.4%)	0/6* (0%)
DENV NS1+, IgM-, IgG+	1/14 (7%)	0/6* (0%)
DENV NS1-, IgM+, IgG+	0/14 (0%)	0/6* (0%)
DENV NS1-, IgM-, IgG+	5/14 (35.7%)	2/6* (33.3%)
DENV NS1+, IgM+, IgG-	5/14 (35.7%)	0/6* (0%)
DENV NS1+, IgM-, IgG-	0/14 (0%)	0/6* (0%)
DENV NS1-, IgM+, IgG-	0/14 (0%)	0/6* (0%)
DENV NS1-, IgM-, IgG-	0/14 (0%)	4/6* (66.7%)

Table 5.3: Sensitivity, specificity, positive (PPV) and negative (NPV) predictive values for PanBio DENV NS1 ELISA when compared to detection of DENV RNA via qRT-PCR. Note that the 'true negative' value of n=7 was left to stand in order to prevent skewing calculations in favor of qRT-PCR even though both assays failed to diagnose dengue correctly in 2 IgG+ samples.

	DENV qRT-PCR+ (n=14)	DENV qRT-PCR- (n=7)	
DENV NS1 ELISA+	9	0	**PPV=100%**
DENV NS1 ELISA-	5	7	**NPV=58.3%**
	Sensitivity= 64.3%	**Specificity= 100%**	

CHAPTER 5. CONCLUSIONS

5.1. Major Points

Due to the somewhat discrete nature of the Chapters detailed in the main body of the text, this section is included to summarize the main conclusions and remaining needs of the study.

Point 1: The MIA shows promise for becoming a useful platform for both **diagnostic and prognostic** purposes with respect to DENV infections.
Specifically: Despite mixed results, DENV NS1, IL-10, MCP-1, and IP-10 should be included in future developments. NS1 because of its successful use in other similar assays (ELISA) and the latter 3 because of their increased detection in DENV infected individuals over those that are healthy. Other potential analytes that should merit further investigation due to our results include: IL-6, IL-8, IFN-γ, VEGF, and RANTES.

Point 2: The ability to multiplex at a level that other techniques cannot currently provide increases the utility of the MIA as a singular assay.
Specifically: This can only increase the potential diagnostic value of the assay as other markers are included, such as: IgG, IgM, IgA, and DENV RNA. We also conclude that DENV NS1 remains as a possible and most important candidate analyte for development and inclusion in MIA.

Point 3: A suitable minimum number of host markers must be identified that provide robust results in identifying patients that will proceed to severe DENV disease from those that will not.
Specifically: IL-10, MCP-1, and IP-10 may not prove to be adequate in order to differentiate disease outcome especially considering the lack of known severe cases in our data sets. Here other potential candidates again include: IL-6, IL-8, IFN-γ, VEGF, and RANTES.

Point 4: The results of this study as well as others like it *demand* that larger sample sizes are collected prior to defining differential immunological profiles.

Point 5: The MIA may also be useful for elucidating pathogenesis using *in vitro* models.
Specifically: Here we suggest that the cell lines used within this study and their response to DENV is investigated using a co-culture model where u937 includes treatments of uninfected and both ADE and non-ADE models of DENV infection prior to co-culturing with HPMEC ST1.6R. We then propose that 27-plex MIA should be used to determine cellular responses, possibly in conjunction with fluorescent labeling and confocal microscopy of co-cultures. Time points should be decreased and replicates should dramtically increase from n=2 reported here.

5.2. Future Goals

Again, we feel that the results obtained here merit additional consideration and we fully encourage the further development of the DENV MIA. The continuation of the project would first rely upon the acquisition of an appropriate number and type of human sera. At a minimum, sera must be obtained from healthy individuals and acutely DENV infected individuals. Regarding DENV virus infected individuals, the samples may be obtained from individuals that have developed warning signs for developing severe illness but this information must be available for analyses. The ideal samples, however, would be obtained from patients that have yet to develop warning signs, in other words early presentations, and come from populations that result in either uncomplicated illness or severe manifestations. This would most appropriately highlight both the early diagnostic and potential prognostic value of the DENV MIA. Potential collaborations with groups in Florida, Puerto Rico, and/or Malaysia can provide these samples. Obtaining samples from multiple populations may also elucidate regional differences in results. The next stage of development would likely consist of 3 stages. First, the continuation of 27-plex screening of sera for the identification of markers indicitave of individual disease outcome. Second, subjecting sera from healthy or actuely DENV-infected individuals to an MIA that

would support the results reported here. This MIA would include markers for IL-10, IP-10, and MCP-1. Our hypothesis is that we would expect a 2-fold elevation of IP-10 and MCP-1 concentrations in severe forms of DENV illness over uncomplicated dengue significant ($p<0.05$) and therefore predicitive for disease severity. We would also expect the converse to be true for IL-10 concentrations in these specific populations of DENV-infected individuals. During this early range of patient presentation, we expect a 2-fold elevation of IL-10 concentrations to be predicitive of uncomplicated disease resolution over those that proceed to severe illness ($p<0.05$). So using our data reported here of a mean concentration of 86.75pg/mL for IL-10 in DENV+ individuals (uncomplicated illness) with a standard deviation of 96 applied to both groups, and applying this hypothesis, we find that we would need a total sample size of at least 212, equally distributed, with an observed effect size of 0.45 and $\alpha=0.05$ in a one-tailed t-test (G*Power v3.1.9.2, Franz Faul, Universitat Kiel, Germany). The third part of future investigations would include the completion of the DENV diagnostic component of the MIA. We believe that our failure to achieve this goal was due to the similarities between the monoclonal antibodies used for capture and detection and we require an appropriate antibody for detection. In this respect, we would advise to search for a previously characterized and already available mAb, that also maps to the same region (within the major antigenic 'wing domain') as our capture antibody but at a different sequence of amino acids. Once this is achieved the multiplexed assay (from 3-plex to 4-plex) can be investigated for potential losses in sensitivity. Completion of the first goal may allow for additional development and optimization of prognostic ability of the DENV MIA. Completion of the second and third goals would achieve the main goal of providing for a diagnostic assay for DENV that is also predictive according to disease severity.

SELECTED REFERENCES AND BIBLIOGRAPHY

Acosta, E.G., V. Castilla, and E.B. Damonte. 2009. Alternative infectious entry pathways for dengue virus serotypes into mammalian cells. Cellular Microbiology. **doi:10.1111/j.1462-5822.2009.01345.x**

Adalja, A.A., T.K. Sell, N. Bouri, and C. Franco. 2012. Lessons learned during the dengue outbreaks in the United States, 2001-2011. EID. **18(4):**608-614.

Aguilar-Setien, A., M.L. Romero-Almaraz, C. Sanchez-Hernandez, R. Figueroa, L.P. Juarez-Palma, M.M. Garcia-Flores, C. Vazquez-Salinas, M. Salas-Rojas, A.C. Hidalgo-Martinez, S. Aguilar Pierle, C. Garcia-Estrada, and C. Ramos. 2008. Dengue virus in Mexican bats. Epidemiol. Infect. **doi:10.1017/S0950268808000460.**

Akey, D.L., W.C. Brown, S. Dutta, J. Konwerski, J. Jose, T.J. Jurkiw, J. DelProposto, C.M. Ogata, G. Skiniotis, R.J. Kuhn, and J.L. Smith. 2014. Flavivirus NS1 crystal structures reveal a surface for membrane association and regions of interaction with the immune system. Science. **343(6173):**881-885. doi:10.1126/science.1247749.

Alcala, A.C., R. Hernandez-Bravo, F. Medina, D.S. Coll, J.L. Zambrano, R.M. del Angel, and E. Ludert. 2017. The dengue virus non-structural protein 1 (NS1) is secreted from infected mosquito cells via a non-classical caveolin-1-dependent pathway (Abstract). J. Gen. Virol. **98(8):**2088-2099. doi:10.1099/jgv.0.000881.

Alcon, S., A. Talarmin, M. Debruyne, A. Falconar, V. Deubel, and M. Flamand. 2002. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J. Clin. Microbiol. **40(2):**376-381. doi:10.1128/JCM.40.2.376-381.2002.

Alen, M.M.F., S.J.F. Kaptein, T. De Burghgraeve, J. Balzarini, J. Neyts, and D. Schols. 2009. Antiviral activity of carbohydrate-binding agents and the role of DC-SIGN in dengue virus infection. Virology. **387:**67-75.

Alhoot, M.A., S.M. Wang, and S. Devi Sekaran. 2011. Inhibition of dengue virus entry and multiplication into monocytes using RNA interference. PLoS Neg. Trop. Dis. **5(11):**1-10 doi:10.1371/journal/pntd.0001410.

Altshuler, M.L. 2006. PCR troubleshooting the essential guide. Caister Academic Press. Norfolk, U.K.

Amarasinghe, A. and G.W. Letson. 2012. Dengue in the Middle East: a neglected, emerging disease of importance. Trans. Royal Soc. Trop. Med. Hyg. **106:**1-2.

Amarasinghe, A., J.N. Kuritsky, G.W. Letson, and H.S. Margolis. 2011. Dengue virus infection in Africa. EID. **17(8):**1349-1354.

Ambrose, J.H., S.D. Sekaran, and A. Azizan. 2017. Dengue virus NS1 protein as a diagnostic marker: commercially available ELISA and comparison to qRT-PCR and serological diagnostic assays currently used by the state of Florida. J. Trop. Medicine. **https://doi.org/10.1155/2017/8072491**.

Ambrose, J.H., L.M. Stark, J.S. Mateus, K.A. Fitzpatrick, and A. Azizan. 2016. Immunological profiles of human sera as determined by microsphere immunoassay (MIA) demonstrates that IL-10, IP-10, and MCP-1 levels are elevated in acute dengue infections. Microbiology and Virology. (Institute of Microbiology and Virology; Astana, Kazakhstan). **2(13):**29-41**.**

Ambrose, J.H., K.A. Fitzpatrick, L.M. Stark, and A. Azizan. 2010. A characterization of the cytokine response in a monocytic U937 cell-line using a 27-plex microsphere-based immunoassay (MIA). ASM 110th General Meeting, San Diego, CA. (presented by self).

Anders, K.L., N.M. Nguyet, N.T.H. Quyen, T.V. Ngoc, T.V. Tram, T.T. Gan, N.T. Tung, N.T. Dung, N.V.V. Chau, B. Wills, and C.P. Simmons. 2012. An evaluation of dried blood spots and oral swabs as alternative specimens for the diagnosis of dengue and screening for past dengue virus exposure. Am. J. Trop. Med. Hyg. **87(1):**165-170.

Anez, G., and M. Rios. 2013. Dengue in the United States of America: A worsening scenario? BioMed Res. Int. **doi:10.1155/2013/678645.**

Anez, G., D.A.R. Heisey, L.M. Espina, S.L. Stramer, and M. Rios. 2012. Phylogenetic analysis of dengue virus types 1 and 4 circulating in Puerto Rico and Key West, Florida, during 2010 epidemics. Am. J. Trop. Med. Hyg. **87(3):**548-553.

Appanna, R., S.M. Wang, S.A. Ponnampalavanar, L.C.S. Lum, and S.D. Sekaran. 2012. Cytokine factors present in dengue patient sera induces alterations of junctional proteins in human endothelial cells. Am. J. Trop. Med. Hyg. **87(5):**936-942.

Applied Biosystems, Inc. Revision A. Reconstituting and diluting primers and TaqMan® probes.

Araújo F.M.C., R.S.N. Brilhante, L.P.G. Cavalcanti, M.F.G. Rocha, R.A. Cordeiro, A.C.B. Perdigão, I.S. Miralles, L.C. Araújo, R.M.C. Araújo, E.G. Lima, and J.J.C. Sidrim. 2011. Detection of the dengue non-structural 1 antigen in cerebral spinal fluid samples using a commercially available enzyme-linked immunosorbent assay. J. Virol. Meth. **177:**128-131.

Arya, S.C., N. Agarwal, S.C. Parikh, and S. Agarwal. 2011. Simultaneous detection of dengue NS1 antigen, IgM plus IgG and platelet enumeration during an outbreak. SQU Med. J. **11(4):**470-476.

Ashour, J., M. Laurent-Rolle, P.Y. Shi, and A. Garcia-Sastre. 2009. NS5 of dengue virus mediates STAT2 binding and degradation. J. Virol. **83(11):**5408-5418.

Avila-Aguero, M.L., C.R. Avila-Aguero, S.L. Um, A. Soriano-Fallas, A. Canas-Coto, and S.B. Yan, 2004. Systemic host inflammatory and coagulation response in the dengue virus primo-infection. Cytokine. **27:**173-179.

Avirutnan, P., R.E. Hauhart, M.A. Marovich, P. Garred, J.P. Atkinson, and M.S. Diamond. 2011. Complement-mediated neutralization of dengue virus requires mannose-binding lectin. mBio. **2(6):**e00276-11. doi:10.1128/mBio.00276-11.

Avirutnan, P., L. Zhang, N. Punyadee, A. Manuyakorn, C. Puttikhunt, W. Kasinrerk, P. Malasit, J.P. Atkinson, and M.S. Diamond. 2007. Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparin sulfate and chondroitin sulfate E. PLoS Pathogens. **3(11):**e183. doi:10.1371/journal.ppat.0030183.

Avirutnan P., N. Punyadee, S. Noisakran, C. Komoltri, S. Thiemmeca, K. Auethavornanan, A. Jairungsri, R. Kanlaya, N. Tangthawornchaikul, C. Puttikhunt, S.N. Pattanakitsakul, P.T. Yenchitsomanus, J. Mongkolsapaya, W. Kasinrerk, N. Sittisombut, M. Husmann, M. Blettner, S. Vasanawathana, S. Bhakdi, and P. Malasit. 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis. **93(8):**1078-88.

Azar, G.J., J.O. Bond, G.L. Chappell, and A.H. Lawton. Follow-up studies of St. Louis encephalitis in Florida. Ann. Intern. Med. **63(2):**212-220.

Aziz, S., R. Ngui, Y.A.L. Lim, I. Sholehah, J. Nur Farhana, A.S. Azizan, and W.S. Wan Yusoff. 2012. Spatial pattern of 2009 dengue distribution in Kuala Lumpur using GIS application. Tropical Biomedicine. **29(1):**113-120.

Azizan, A., J.H. Ambrose, K.A. Fitzpatrick, and L.M. Stark. 2010. A cytokine MIA for severe flavivirus infections. 8th Annual ASM Biodefense and Emerging Diseases Research Meeting, Baltimore, MD. (presented by A. Azizan).

Azizan, A., K.A. Fitzpatrick, A. Signarovitz, R. Tanner, H. Hernandez, L.M. Stark, and D. Kazanis. 2009. Profile of time-dependent VEGF upregulation in human pulmonary endothelial cells, HPMEC-ST1.6R infected with DENV-1, -2, -3, and -4 viruses. Virol. J. **6:**49. doi:10.1186/1743-422X-6-49.

Azizan, A., J. Sweat, C. Espino, J. Gemmer, L.M. Stark, and D. Kazanis. 2006. Differential proinflammatory and angiogenesis-specific cytokine production in human pulmonary endothelial cells, HPMEC-STR1.6R infected with dengue-2 and dengue-3 virus. J. Viro. Met. **138:**211-217.

Balsitis, S.J. and E. Harris. 2010. Animal models of dengue virus infection and disease: applications, insights, and frontiers, p. 103-120. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Barrera, R., A.M. Bingham, H.K. Hassan, M. Amador, A.J. Mackay, and T.R. Unnasch. 2012. Vertebrate hosts of Aedes aegypti and Aedes mediovittatus (Diptera: Culicidae) in rural Puerto Rico. J. Med. Entom. **49(4):**917-921.

Barrow, A.D. and J. Trowsdale. 2006. You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signaling. Eur. J. Immunol. **36:**1646-1653.

Barzon, L., M. Pacenti, E. Franchin, S. Pagni, E. Lavezzo, L. Squarzon, T. Martello, F. Russo, L. Nicoletti, G. Rezza, C. Castilletti, M.R. Capobianchi, P. Salcuni, M. Cattai, R. Cusinato, and G. Palu. 2013. Large human outbreak of West Nile virus infection in north-eastern Italy in 2012. Viruses. **5:**2825-2839. doi:10.3390/v5112825.

Basile, A.J., K. Horiuchi, A.J. Panella, J. Laven, O. Kosoy, R.S. Lanciotti, N. Venkateswaran, and B.J. Biggerstaff. 2013. Multiplex microsphere immunoassays for the detection of IgM and IgG to arboviral diseases. PLoS ONE. **8(9):e75670.** doi:10.1371/journal.pone.0075670.

Basile, A.J., B.J. Biggerstaff, O.L. Kosoy, S.R. Junna, N.A. Panella, A.M. Powers, L.M. Stark, and N.M. Nemeth. 2010. Removal of species constraints in antibody detection. Clin. Vaccine Immunol. **17(1):**56-61.

Basu, A. and U.C. Chaturvedi. 2008. Vascular endothelium: the battlefield of dengue viruses. FEMS Immunol. Med. Microbiol. **1:**1-13.

Beasley, D.W.C. and A.D.T. Barrett. 2010. The infectious agent, p.29-73. In S.B. Halstead (ed.), Dengue. Imperial College Press, London, U.K.

Becker, N., D. Petric, M. Zgomba, C. Boase, C. Dahl, J. Lane, and A. Kaiser. 2003. IV. Control of mosquitoes (ebook). In Mosquitoes and their control. Kluwer Academic/Plenum Publishers, New York, New York.

Beg, A.A., and X. Wang. 2009. The nuclear factor-κB transcription factor pathway, p.107-118. In A.R. Brasier, A. Garcia-Sastre, and S.M. Lemon (ed.). Cellular signaling and innate immune responses to RNA virus infections. ASM Press, Washington, D.C.

Belinato, T.A., A.J. Martins, and D. Valle. 2012. Fitness evaluation of two Brazilian Aedes aegypti field populations with distinct levels of resistance to the organophosphate temephos. Mem. Inst. Oswaldo Cruz. **107(7):**916-922.

Bennett, S.N. 2010. Evolutionary dynamics of dengue virus, p. 157-172. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Bessoff, K., E. Phoutrides, M. Delorey, L. Nereida Acosta, and E. Hunsperger. 2010. Defining the utility of a commercial NS1 antigen capture kit as a dengue virus (DENV) diagnostic tool. Clin. Vaccine Immunol. **doi:10.1128/CVI.00041-10.**

Bhatt, S., P.W. Gething, O.J. Brady, J.P. Messina, A.W. Farlow, C.L. Moyes, J.M. Drake, J.S. Brownstein, A.G. Hoen, O. Sankoh, M.F. Myers, D.B. George, T. Jaenisch, G.R.W. Wint, C.P. Simmons, T.W. Scott, J.J. Farrar, and S.I. Hay. 2013. The global distribution and burden of dengue. Nature. **doi:10.138/nature12060.**

Bio-Rad. 2010. Bio-Rad receives notification from the FDA that it may proceed with an investigational new drug study for its dengue NS1 Ag microplate assay. http://www.bio-rad.com/en-us/corporate/newsroom/bio-rad-receives-notification-from-the-fda-that-it-may-proceed-with-an-investigational-new-drug-study-for-its-dengue-ns1-ag-microplate-assay?email_icon=3_click_email&custom_event=Email%20Icon%20-%20Email%20Clickthrough. Bio-Rad Laboratories, Inc., Hercules, CA.

Bio-Rad. Micro Bio-Spin™ chromatography columns, instruction manual, revision C. Bio-Rad Laboratories, Inc., Hercules, CA.

Bio-Rad. 2010. Bio-Plex Pro™ magnetic COOH beads amine coupling kit, instruction manual, revision C. Bio-Rad Laboratories, Inc., Hercules, CA.

Bio-Rad. 2008. Bio-Plex Pro™ assays: cytokine, chemokine, and growth factors, instruction manual, revision A. Bio-Rad Laboratories, Inc., Hercules, CA.

Blacksell, S.D., M.P. Mammen, Jr., S. Thongpaseuth, R.V. Gibbons, R.G. Jarman, K. Jenjaroen, A. Nisalak, R. Phetsouvanh, P.N. Newton, and N.P.J. Day. 2008. Evaluation of the Panbio dengue virus nonstructural 1 antigen detection and immunoglobulin M antibody enzyme-linked immunorsorbent assays for the diagnosis of acute dengue infections in Laos. Diag. Microbiol. Infect. Dis. **60:**43-49.

Blank, U., P. Launay, M. Benhamou, and R.C. Monteiro. 2009. Inhibitory ITAMs as novel regulators of immunity. Immunological Reviews. **232:**59-71.

Block, O.K.T., S.I.R.W.W. Rodrigo, M. Quinn, X. Jin, R.C. Rose, and J.J. Schlesinger. 2010. A tetravalent recombinant dengue domain III protein vaccine stimulates neutralizing and enhancing antibodies in mice. Vaccine. **doi:10.1016/j.vaccine.2010.10.004.**

Bogdan, C. 2009. Regulation and anti-microbial function of inducible nitric oxide synthase in phagocytes, p. 367-378. In D.G. Russell and S. Gordon (ed.), Phagocyte-pathogen interactions: macrophages and the host response to infection. ASM Press, Washington D.C.

Boonak, K., K.M. Dambach, G.C. Donofrio, B. Tassaneetrithep, and M.A. Marovich. 2010. Cell type specificity and host genetic polymorphisms influence antibody dependent enhancement of dengue virus infection. J. Virol. **doi:10.1128/JVI.00220-10.**

Boonnak, K., B.M. Slike, T.H. Burgess, R.M. Mason, S.J. Wu, P. Sun, K. Porter, I.F. Rudiman, D. Yuwono, P. Puthavathana, and M.A. Marovich. 2008. Role of dendritic cells in antibody dependent enhancement of dengue infection. J. Virol. **82(8):**3939-3951.

Bosch, I., K. Xhaja, L. Estevez, G. Raines, H. Melichar, R.V. Warke, M.V. Fournier, F.A. Ennis, and A.L. Rothman. 2002. Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. J. Virol. **76:**5588-5597.

Brasier, A.R. 2009. The nuclear factor-κB signaling network: insights from systems approaches, p.119-135. In A.R. Brasier, A. Garcia-Sastre, and S.M. Lemon (ed.). Cellular signaling and innate immune responses to RNA virus infections. ASM Press. Washington, D.C.

Brathwaite Dick, O., San Matin, J.L., Montoya, R.H., del Diego, J., Zambrano, B., and G.H. Dayan. 2012. Review: the history of dengue outbreaks in the Americas. Am. J. Trop. Med. Hyg. **87(4)**:584-593.

Brien, J.D., S.K. Austin, S. Sukupolvi-Petty, K.M. O'Brien, S. Johnson, D.H. Fremont, and M.S. Diamond. 2010. Genotype-specific neutralization and protection by antibodies against dengue virus type 3. J. Virol. **84(20):**10630-10643.

Briese, T., X.Y. Jia, C. Huang, L.J. Grady, and W.I. Lipkin. 1999. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. Lancet. **354:**1261-1262.

Buchy, P and R. Peeling. 2009. Chapter 4: laboratory diagnosis and diagnostic tests, p.91-110. In Dengue: guidelines for diagnosis, treatment, prevention, and control. WHO Press, Geneva, Switzerland.

Buckner, E.A., B.W. Alto, and L.P. Lounibos. 2013. Vertical transmission of Key West dengue-1 virus by Aedes aegypti and Aedes albopictus (Diptera: Culicidae) mosquitoes from Florida (Abstract-ahead of publication). J. Med. Entomol. **50(6):**1291-1297.

Bugrysheva, J.V., V.A. Matveeva, E.Yu. Dobrikova, N.V. Bykovskaya, S.A. Korobova, V.N. Bakhvalova, O.V. Morozova. 2001. Tick-borne encephalitis virus NS1 glycoprotein during acute and persistent infection of cells. Vir. Res. **76:**161-169.

Calisher, C.H., Karabatsos, N., Dalrymple, J.M., Shope, R.E., Porterfield, J.S., Westaway, E.G., and W.E. Brandt. 1989. Antigenic relationships between Flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol. **70:**37-43.

Carvalho, D.O., A.R. McKemey, L. Garziera, R. Lacroix, C.A. Donnelly, L. Alphey, A. Malavasi, and M.L. Capurro. 2015. Suppression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes. PLoS Neg. Trop. Dis. **9(7):**e0003864. doi:10.1371/journal.pntd.0003864.

Caprara, A., J. Wellington de Oliveira Lima, A. Correia Pequeno Marinho, P. Gondim Calvasina, L. Paes Landim, J. Sommerfeld. 2009. Irregular water supply, household usage and dengue: a bio-social study in the Brazilian northeast. Cad. Saude Publica, Rio de Janeiro. **25(sup):**S125-S136.

Castro-Jorge, L.A., P.R.L Machado, C.A. Favero, M.C. Borges, L.M.R. Passos, R. Moreira de Oliveira, and B.A.L. Fonseca. 2010. Clinical evaluation of the NS1 antigen-capture ELISA for early diagnosis of dengue virus infection in Brazil. J. Med. Virol. **82:**1400-1405.

Cecilio, A.B., E.S. Campanelli, K.P.R. Souza, L.B. Figueiredo, and M.C. Resende. 2009. Natural vertical transmission by Stegomyia albopicta as dengue vector in Brazil. Braz. J. Biol. **69(1):**123-127.

Centers for Disease Control (CDC). 2017. 2017 case counts in US. https://www.cdc.gov/zika/reporting/2017-case-counts.html. Accessed 11/10/2017.

Centers for Disease Control (CDC). 2017. 2016 case counts in US. https://www.cdc.gov/zika/reporting/2016-case-counts.html. Accessed 11/10/2017.

Centers for Disease Control (CDC). 2010. Locally-acquired dengue-Key West, Florida, 2009-2010. MMWR. **59(19):**577-581.

Chan, K.R., S.L.X. Zhang, H.C. Tan, Y.K. Chan, A. Chow, A.P.C. Lim, S.G. Vasudevan, B.J. Hanson, and E.E. Ooi. 2011. Ligation of Fc gamma receptor IIB inhibits antibody-dependent enhancement of dengue virus infection. PNAS. doi:10.1073/pnas.1106568108/-/DCSupplemental.

Chang, A.Y., M.E. Parrales, J. Jimenez, M.E. Sobieszczyk, S.M. Hammer, D.J. Copenhaver, and R.P. Kulkarni. 2009. Combining Google Earth and GIS mapping technologies in a dengue surveillance system for developing countries. I. J. Health Geographics. **8:49.**

Chang, G.J. 1997. Molecular biology of dengue viruses, p. 175-198. In D.J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International.

Chang, Y., D. Bluteau, N. Debili, and W. Vainchenker. 2007. From hematopoietic stem cells to platelets. J. Thromb. Haemost. **5(supp. 1):**318-327.

Chase, A. F.A. Medina, and J.L. Muñoz-Jordán. 2011. Impairment of CD4+ T cell polarization by dengue virus-infected dendritic cells. JID. **203:**1763-1774.

Chatjeri, S., J.C. Allen, Jr., A. Chow, Y.S. Leo, and E.E. Ooi. 2011. Evaluation of the NS1 rapid test and the WHO dengue classification schemes for use as bedside diagnosis of acute dengue fever in adults. Am. J. Trop. Med. Hyg. **84(2):**224-228.

Chen, L.C., H.W. Shyu, H.M. Lin, H.Y. Lei, Y.S. Lin, H.S. Liu, and T.M. Yeh. 2009. Dengue virus induces thrombomodulin expression in human endothelial cells and monocytes *in vitro*. J. Infect. **58:**368-374.

Chen R. and N. Vasilakis. 2011. Dengue- quo tu et quo vadis? Viruses. **3:**1562-1608.

Chen, S.T., Y.L. Lin, M.T. Huang, M.F. Wu, S.C. Cheng, H.Y. Lei, C.K. Lee, T.W. Chiou, C.H. Wong, and S.L. Hsieh. 2008. CLEC5A is critical for dengue-virus-induced lethal disease. Nature. **453(29):**672-678.

Chen, Y., T. Maguire, and R.M. Marks. 1996. Demonstration of binding of dengue virus envelope protein to target cells. J. Virol. **70(12):**8765-8772.

Chien, L.J., T.L. Liao, P.Y. Shu, J.H. Huang, D.H. Gubler, and G.J.J. Chang. 2005. Development of real-time reverse transcriptase PCR assays to detect and serotype dengue viruses. J. Clin. Microbiol. **44(4):**1295-1304.

Christophers, S.R. 1960. Aedes aegypti the yellow fever mosquito: its life history, binomics, and structure. Cambridge University Press. Cambridge, U.K.

Chua, K.B., I.L. Chua, I.E. Chua, and K.H. Chua. 2008. Effect of chemical fogging on immature Aedes mosquitoes in natural field conditions. Singapore Med. J. **46(11):**639-644.

Chuansumrit, A., W. Chaiyaratana, V. Pongthanapisith, K. Tangnararatchakit, S. Lertwongrath, and S. Yoksan. 2008. The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection. Pediatr. Infect. Dis. J. **27:**43-48.

Chung, K.M. and M.S. Diamond. 2008. Defining the levels of secreted non-structural protein NS1 after West Nile virus infection in cell culture and mice. J. Med. Virol. **80:**547-556.

Chung, K.M., B.S. Thompson, D.H. Fremont, and M.S. Diamond. 2007. Antibody recognition of cell surface-associated NS1 triggers Fc-γ receptor-mediated phagocytosis and clearance of West Nile virus infected cells. J. Virol. **81(17):**9551-9555.

Chung, K.M., G.E. Nybakken, B.S. Thompson, M.J. Engle, A. Marri, D.H. Fremont, and M.S. Diamond. 2006. Antibodies against West Nile virus nonstructural protein NS1 prevent lethal infection through Fc γ receptor-dependent and –independent mechanisms. J. Virol. **80(3):**1340-1351.

Cicmil, M., J.M. Thomas, M. Leduc, C. Ben, and J.M. Gibbons. 2002. Platelet endothelial cell adhesion molecule-1 signaling inhibits the activation of human platelets. Blood. **99(1):**137-144.

Cleland, J.B. and B. Bradley. 1919. Further experiments in the etiology of dengue fever. J. Hyg. **18(3):**217-254.

Clyde, K., J.L. Kyle, and E. Harris. 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J. Virol. **80(23):**11418-11431.

Coelho, G.E., M. Nascimento Burattini, M. da Gloria Teixeira, F.A. Bezerra Coutinho, and E. Massad. 2008. Dynamics of the 2006/2007 dengue outbreak in Brazil. Mem. Inst. Oswaldo Cruz. **103(6):**535-539.

Dalrymple, N.A. and E.R. Mackow. 2012. Roles for endothelial cells in dengue virus infection. Adv. Virol. **doi:10.1155/2012/840654.**

Day, J. and J. Shaman. 2009. Severe winter freezes enhance St. Louis encephalitis virus amplification and epidemic transmission in peninsular Florida. J. Med. Entomol. **46(6):**1498-1506.

Day, J. 2001. Predicting St. Louis encephalitis virus epidemics: lessons from recent, and not so recent, outbreaks. Ann. Rev. Entomol. **46:**111-138.

Day, J.F. and L.M. Stark. 2000. Frequency of St. Louis encephalitis virus in humans from Florida, USA: 1990-1999. J. Med. Entomol. **37(4):**626-633.

Day, J.F. and L.M. Stark. 1996. Transmission patterns of St. Louis encephalitis and eastern equine encephalitis viruses in Florida: 1978-1993. J. Med. Entomol. **33:**132-139.

Day, J.F., L.M. Stark, J.T. Zhang, A.M. Ramsey, and T.W. Scott. 1996. Antibodies to arthropod-borne encephalitis viruses in small mammals from Florida. J. Wildlife Dis. **32(3):**431-436.

de Carvalho Bittencourt, M., J. Martial, A. Cabie, L. Thomas, and R. Cesaire. 2012. Decreased peripheral dendritic cell numbers in dengue virus infection. J. Clin. Immunol. **32:**161-172.

de Figueiredo, R.M.P., F.G. Naveca, C.M. Oliveira, M.S. Bastos, M.P.G. Mourao, S. de S. Viana, M.N. Melo, E.F. Itapirema, E., C.J. Saatkamp, and I.P. Farias. 2011. Co-infection of dengue virus by serotypes 3 and 4 in patients from Amazonas, Brazil. Rev. Inst. Med. Trop. Sao Paulo. **53(6):**321-323.

de la Cruz-Hernandez, S.I., H. Flores-Aguilar, S. Gonzalez-Mateos, I. Lopez-Martinez, C. Alpuche-Aranda, J.E. Ludert, and R.M. del Angel. 2013. Determination of viremia and concentration of circulating nonstructural protein 1 in patients infected with dengue virus in Mexico. Am. J. Trop. Med. Hyg. **88(3):**446-454. doi:10.4269/ajtmh.12-0023.

de M. Campos, R., C.S.B. Veiga, M.D.F. Meneses, L.M. de Souza, V. Malirat, J.P. Albuquerque, J. Schmidt-Chanasit, and D.F. Ferreira. 2013. Emergence of dengue virus 4 genotypes II b and I in the city of Rio de Janeiro. J. Clin. Virol. **56:**86-88.

de Oliveira Franca, R.F., S. Zucoloto, and B.A.L. da Fonseca. 2010. A BALB/c mouse model show that liver involvement in dengue disease is immune-mediated. Exp. Molec. Pathol. **doi:10.1016/j.yexmp.2010.07.007.**

de Oliveira-Pinto, L.M., M. Gandini, L. Picinini Freitas, M. Mendonça Siqueira, C. Ferreira Marinho, S. Setúbal, C. Fernandes Kubelka, O Gonçalves Cruz, and S.A. de Oliveira. 2012. Profile of circulating levels of IL-1Ra, CXCL10/IP-10, CCL4/MIP-1β, and CCL2/MCP-1 in dengue fever and parvovirosis. Mem. Inst. Oswaldo Cruz. **107(1):**48-56.

Deen, J., L. Lum, E. Martinez, and L.H. Tan. 2009. Chapter 2: clinical management and delivery of clinical services, p.25-58. In Dengue: guidelines for diagnosis, treatment, prevention, and control. WHO Press, Geneva, Switzerland.

Degreve, L., C.A. Fuzo, and A. Caliri. 2012. Extensive structural change of the envelope protein of dengue virus induced by a tuned ionic strength: conformational and energetic analyses. J. Comput. Aided Mol. Des. **26:**1311-1325.

Dejnirattisai, W., T. Duangchinda, C.L.S. Lin, S. Vasanawathana, M. Jones, M. Jacobs, P. Malasit, X.N. Xu, G. Screaton, and J. Mongkolsapaya. 2008. A complex interplay among virus, dendritic cells, T cells, and cytokines in dengue virus infections. J. Immunol. **181:**5865-5874.

Dengue consensus 2003. 2003. Clinical practice guidelines: dengue infection in adults. Academy of Medicine Malaysia, Ministry of Health**.**

DengueMAP. 2014. http://www.healthmap.org/dengue/en/

Dewi, B.E., T. Takasaki, and I. Kurane. 2004. *In vitro* assessment of human endothelial cell permeability: effects of inflammatory cytokines and dengue virus infection. J. Viro. Met. **121:**171-180.

Diamond, M.S. and R.S. Klein. 2004. West Nile virus: crossing the blood-brain barrier. Nature Med. **10(12):**1294-1295.

Diamond, M.S., D. Edgil, T.G. Roberts, B. Lu, and E. Harris. 2000. Infection of human cells by dengue virus is modulated by different cell types and viral strains. **74(17):**7814-7823.

Dias, L.L., A.A. Amarilla, T.R. Poloni, D.T. Covas, V.H., and L.T. Figueiredo. 2012. Detection of dengue virus in sera of Brazilian blood donors. Transfusion. **52(8):**1667-1671.

Ding, X.X., D.M. Hu, Y. Chen, B. Di, J. Jin, Y.X. Pan, L.W. Qiu, Y.D. Wang, K. Wen, M. Wang., and X.Y. Che. 2011. Full serotype- and group-specific NS1 capture ELISA for rapid differential diagnosis of dengue infection. Clin. Vacc. Immunol. (ahead of print). **doi:10.1128/CVI.00462-10**.

Dittmar, D., H.G. Haines, and A. Castro. 1980. Monoclonal antibodies specific for dengue virus type 3. J. Clin. Microbiol. **12(1):**74-78.

Dobson, M. 2007. Disease the extraordinary stories behind history's deadliest killers. Quercus, London, U.K. 152-155.

Dong, T., E. Moran, N. Vinh Chau, C. Simmons, K. Luhn, Y. Peng, B. Wills, N.P. Dung, L.T.T. Thao, T.T. Hien, A. McMichael, J. Farrar, and S. Rowland-Jones. 2007. High pro-inflammatory cytokine secretion and loss of high avidity cross-reactive cytotoxic T-cells during the course of secondary dengue virus infection. PLoS ONE. **12**(e1192):1-12. doi:10.1371/journal.pone.0001192.

dos Santos, F.B., F.B. Nogueira, M.G. Castro, P.C.G. Nunes, A.M.B. de Filippis, N.R.C. Faria, J.B.S. Simoes, S.A. Sampaio, C.R. Santos, and R.M.R. Nogueira. 2011. First report of multiple lineages of dengue viruses type 1 in Rio de Janeiro, Brazil. Virology J. **8:387.**

Duangchinda, T., W. Dejnirattisai, S. Vasanawathana, W. Limpitikul, N. Tangthawornchaikul, P. Malasit, J. Mongkolsapaya, and G. Screaton. 2010. Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. PNAS. **107(39):**16922-16927.

Dunbar, M.R., M.W. Cunningham, and J.C. Roof. 1998. Seroprevalence of selected disease agents from free-ranging black bears in Florida. J. Wildlife Dis. **34(3):**612-619.

Dussart, P., B. Labeau, G. Lagathu, P. Louis, M.R.T. Nunes, S.G. Rodrigues, C. Storck-Herrmann, R. Cesaire, J. Morvan, M. Flamand, and L. Baril. 2006. Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin. Vacc. Immunol. **13(11):**1185-1189.

Duval, C.W. and W.H. Harris. 1924. Study upon the etiology of dengue fever: II. Cultivation and nature of the virus. J. Exp. Med. **40(6):**835-844.

Ehrenrkranz, N.J., W.L. Pond, R.M. Pennington, and M.J. Carter. 1963. Arthropod-borne virus disease in Florida. Report of a 1958 outbreak in Miami and a serologic survey of Miami residents. Am. J. Med. **35:**673-681.

Egger, J.R., E.E. Ooi, D.W. Kelly, M.E. Woolhouse, C.R. Davies, and P.G. Coleman. 2008. Reconstructing historical changes in the force of infection of dengue fever in Singapore: implications for surveillance and control. Bull. WHO. **86:**187-196.

Enserink, M. 2010. GM mosquito trial alarms opponents, strains ties in gate. Science. **330(6007):**1030-1031.

Esteva, L. and C. Vargas. 2000. Influence of vertical and mechanical transmission on the dynamics of dengue disease. Mathematical Biosciences. **167:**51-64.

Fagbami, A., S.B. Halstead, N. Marchette, and K. Larsen. 1988. Heterologous flavivirus infection-enhancing antibodies in sera of Nigerians. Am. J. Trop. Med. Hyg. **38(1):**205-207.

Falconar, A.K.I. and C.M.E. Romero-Vivas. 2011. Simple prognostic criteria can definitively identify patients who develop severe versus non-severe dengue disease, or have other febrile illnesses. J. Clin. Med. Res. **4(1):**33-44.

Falconar, A.K.I. 2008. Monoclonal antibodies that bind to common epitopes on the dengue virus type 2 nonstructural-1 and envelope glycoproteins display weak neutralizing activity and differentiated responses to virulent strains: implications for pathogenesis and vaccines. Clin. Vaccine Immunol. **15(3):**549-561.

Falconar, A.K.I. 2007. Antibody responses are generated to immunodominant ELK/KLE-type motifs on the nonstructural-1 glycoprotein during live dengue virus infections in mice and humans: implications for diagnosis, pathogenesis, and vaccine design. Clin Vaccine Immunol. **14(5):**493-504.

Falconar, A.K.I., E. de Plata, and C.M.E. Romero-Vivas. 2006. Altered enzyme-linked immunosorbent assay immunoglobulin M (IgM)/IgG optical density ratios can correctly classify all primary or secondary dengue virus infections 1 day after the onset of symptoms, when all of the viruses can be isolated. Clin. Vacc. Immunol. **13(9):**1044-1051.

Falconar, A.K., P.R. Young, and M.A. Miles. 1994. Precise location of sequential dengue virus subcomplex and complex B cell epitopes on the nonstructural-1 glycoprotein (*Abstract*). Arch Virol. **137(3-4):**315-326.

Falconar, A.K.I. and P.R. Young. 1991. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1. J. Gen. Virol. **72:**961-965.

Faria Oliveira, M., J.M. Galvao Araujo, O. Costa Ferreira, Jr., D. Fernandes Ferreira, D. Bonfim Lima, F. Barreto Santos, H. Goncalves Schatzmayr, A. Tanuri, and R.M. Ribeiro Nogueira. 2010. Two lineages of dengue virus type 2, Brazil. EID. **16(3):**576-578.

Fiueiredo, M.L.G., A. de C. Gomes, A.A. Amarilla, A. de S. Leandro, A. de S. Orrico, R.F. de Araujo, J. do S.M. Castro, E.L. Durigon, V.H. Aquino, and L.T.M. Figueiredo. 2010. Mosquitoes infected with dengue viruses in Brazil. Virol. J. **7(152).**

Fink, J., F. Gu, and S.G. Vasudevan. 2006. Role of T-cells, cytokines and antibody in dengue fever and dengue haemorrhagic fever. Rev. Med. Virol. **16:**263-275.

Fink, K., C. Ng, C. Nkenfou, S.G. Vasudevan, N. van Rooijen, and W. Schul. 2009. Depletion of macrophages in mice results in higher dengue virus titers and highlights the role of macrophages for virus control. Eur. J. Immunol. **39:**1-13.

Fischl, W. and R. Bartenschlager. 2011. Exploitations of cellular pathways by dengue virus. Curr. Op. Microbiol. **14:**470-475.

Flamand, M., F. Megret, M. Mathieu, J. Lepault, F.A. Rey, and V. Deubel. 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J. Virol. **73(7):**6104-6110.

Fleeton, M.N., B.J. Sheahan, E.A. Gould, G.J. Atkins, and P. Liljeström. 1999. Recombinant Semliki Forest virus particles encoding the prME or NS1 proteins of louping ill virus protect mice from lethal challenge. J. Gen. Virol. **80:**1189-1198.

Fleming, I. 2010. Molecular mechanisms underlying the activities of eNOS. Eur. J. Physiol. **459:**793-806.

Flores-Mendoza, L.K., T. Estrada-Jiminez, V. Sedeno-Monge, M. Moreno, M.C. Manjarrez, G. Gonzalez-Ochoa, L.M.P. Pena, and J. Reyes-Leyva. 2017. IL-10 and socs3 are predictive biomarkers of dengue hemorrhagic fever. Med. Inflamm. https://doi.org/10.1155/2017/5197592.

Florida Health. 2016. Health officials issue mosquito-borne illness advisory. http://www.floridahealth.gov/diseases-and-conditions/mosquito-borne-diseases/_documents/2016/monroe-dengue-5-31-16.pdf.

Florida Health. 2015. Dengue Fever- dengue occurrence in Florida. http://www.floridahealth.gov/diseases-and-conditions/dengue/.

Florida Keys Mosquito Control District. 2014. Florida Keys mosquito control- our history. http://keysmosquito.org/history/.

Forsstrom, B., B.B. Axnas, J. Rockberg, H. Danielsson, A. Bohlin, and M. Uhlen. 2015. Dissecting antibodies with regards to linear and conformational epitopes. PLoS One. **10(3):**e0121673. doi:10.1371/journal.pone.0121673.

Freshney, R.I. 2010. Chapter 1 Introduction, p.1-6. In, Culture of animal cells, a manual of basic technique and specialized applications, 6th Ed. John Wiley and Sons, Inc. Hoboken, N.J.

Frost, M.J., J. Zhang, J.H. Edmonds, N.A. Prow, X. Gu, R. Davis, C. Hornitzky, K.E. Arzey, D. Finlaison, P. Hick, A. Read, J. Hobson-Peters, F.J. May, S.L. Doggett, J. Haniotis, R.C. Russell, R.A. Hall, A.A. Khromykh, and P.D. Kirkland. 2011. Characterization of virulent West Nile virus Kunjin strain, Australia, 2011. EID. **18(5):**doi:10.3201/eid1805.111720.

Fuchs, I., H. Bin, S. Schlezinger, and E. Schwartz. 2014. NS1 antigen testing for the diagnosis of dengue in returned Israeli travelers. J. Med. Virol. **doi:10.1002/jmv.23879.**

Galvao de Araujo, J.M., G. Bello, H. Romero, and R.M. Ribeiro Nogueira. 2012. Origin and evolution of dengue virus type 3 in Brazil. PLoS Neg. Trop. Dis. **6(9):**e1784.

Gaikwad, S., S.S. Sawant, and J.S. Shastri. 2017. Comparison of nonstructural protein-1 antigen detection by rapid and enzyme-linked immunosorbent assay test and its correlation with polymerase chain reaction for early diagnosis of dengue. J. Lab. Physicians. **9(3):**177-181. doi:10.4103/0974-2727.208265.

Gamarnik, A. 2010. Role of the dengue virus 5' and 3' untranslated regions in viral replication, p.55-76. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Garg, R.K., H.S. Malhotra, A. Gupta, N. Kumar, and A. Jain. 2012. Concurrent dengue virus and Japanese encephalitis virus infection of the brain: is it co-infection or co-detection? Infection. **doi:10.1007/s15010-012-0284-z.**

Gaunt, M.W. A.A. Sall, X. de Lamballerie, A.K.I. Falconar, T.I. Dzhivanian, and E.A. Gould. 2001. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association, and biogeography. J. Gen. Virol. **82:**1867-1876.

Gibbons, R.V., M. Streitz, T. Babina, and J.R. Fried. 2012. Dengue and US military operations from the Spanish-American War through today. EID. **18(4):**623-630.

Goldblum, N., V.V. Sterk, and B. Paderski. 1954. The clinical features of the disease and the isolation of West Nile virus from the blood of nine human cases. Am. J. Hyg. **59:**89-103.

Goncalvez, A.P., R.H. Purcell, and C.J. Lai. 2010. Progress in passive immunotherapy, p. 265-297. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Goncalves de Castro, M., F. Bruycker de Nogueira, R.M. Ribeiro Nogueira, R. Lourenco-de-Oliveira, and F. Barreto dos Santos. Genetic variation in the 3' untranslated region of dengue virus serotype 3 strains isolated from mosquitoes and humans in Brazil. Virol. J. **10(3):**1-11.

Gorman, K., J. Young, L. Pineda, R. Marquez, N. Sosa, D. Bernal, R. Torres, Y. Soto, R. Lacroix, N. Naish, P. Kaiser, K. Tepedino, G. Philips, C. Kosmann, and L. Caceres. 2015. Short-term suppression of Aedes aegypti using genetic control does not facilitate Aedes albopictus. Pest Manag. Sci. **72:**618-628. doi:10.1002/ps.4151.

Goswami, R.P., A. Mukherjee, T. Biswas, P.S. Karmakar, and A. Ghosh. 2012. Two cases of dengue meningitis: a rare first presentation. J. Infect. Dev. Ctries. **6(2):**208-211.

Gould, E.A. and A. Buckley. 1989. Antibody-dependent enhancement of yellow fever and Japanese encephalitis virus neurovirulence. J. Gen. Virol. **70:**1605-1608.

Green, S. and A. Rothman. 2006. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr. Opin. Infect. Dis. **19:**429-436.

Green, W.R. 2004. Cell-mediated immunity, p. 373-398. In G.B. Pier, J.B. Lyczak, and L.M. Wetzler (ed.). Immunology, infection, and immunity. ASM Press. Washington, D.C.

Grubaugh, N.D., J.T. Ladner, M.U.G. Kraemer, G. Dudas, A.L. Tan, K. Gangavarapu, M.R. Wiley, S. White, J. Thézé, D.M. Magnani, K. Prieto, D. Reyes, A.M. Bingham, L.M. Paul, R. Robles-Sikisaka,

G. Oliveira, D. Pronty, C.M. Barcellona, H.C. Metsky, M.L. Baniecki, K.G. Barnes, B. Chak, C.A. Freije, A. Gladden-Young, A. Gnirke, C. Luo, B. MacInnis, C.B. Matranga, D.J. Park, J. Qu, S.F. Schaffner, C. Tomkins-Tinch, K.L. West, S.M. Winnicki, S. Wohl, N.L. Yozwiak, J. Quick, J.R. Fauver, K. Khan, S.E. Brent, R.C. Reiner Jr, P.N. Lichtenberger, M.J. Ricciardi, V.K. Bailey, D.I. Watkins, M.R. Cone, E.W. Kopp IV, K.N. Hogan, A.C. Cannons, R. Jean, A.J. Monaghan, R.F. Garry, N.J. Loman, N.R. Faria, M.C. Porcelli, C. Vasquez, E.R. Nagle, D.A.T. Cummings, D. Stanek, A. Rambaut, M. Sanchez-Lockhart, P.C. Sabeti, L.D. Gillis, S.F. Michael, T. Bedford, O.G. Pybus, S. Isern, G. Palacios, and K.G. Andersen. 2017. Genomic epidemiology reveals multiple introductions of Zika virus into the United States. Nature **546:**401–405. doi:10.1038/nature22400

Gubler, D.J. 2007. The continuing spread of West Nile virus in the Western Hemisphere. Clin. Infect. Dis. **45:**1039-1046.

Gubler, D.J. 1997. Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem, p.1-22. In D.J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International.

Gulati, S. and A. Maheshwari. 2007. Atypical manifestations of dengue. Trop. Med. Int. Health. **12(9):**1087-1095.

Gullard, A. 2013. Burden of dengue fever is higher than previously thought. BMJ. **347:**f6280. doi:10.1136/bmj.f6280.

Gupta, M., A. MacNeil, Z.D. Reed, P.E. Rollin, and C.F. Spiropoulou. 2012. Serology and cytokine profiles infected with the newly discovered Bundibugyo ebolavirus. Virology. **423:**119-124.

Gutsche, I., F. Coulibaly, J.E. Voss, J. Salmon, J. d'Alayer, M. Ermonval, E. Larquet, P. Charneau, T. Krey, F. Mégret, E. Guittet, F.A. Rey, and M. Flamand. 2011. Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. PNAS. **108(19):**8003-8008.

Guzman, M.G., B. Sierra, G. Kouri, J. Farrar, and C. Simmons. 2010. Host and virus determinants of susceptibility and dengue disease severity, p. 79-101. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Guzman, M.G. 2005. Deciphering dengue: the Cuban experience. Science. **309:**1495-1497.

Hall, R.A., A.A. Khromykh, J.M. Mackenzie, J.H. Scherret, T.I. Khromykh, and J.S. Mackenzie. 1999. Loss of dimerisation of the nonstructural protein NS1 of Kunjin virus delays viral replication and reduces virulence in mice, but still allows secretion of NS1. Virology. **264:**66-75.

Halstead SB. 2010. Pathophysiology, p.285-326. In Halstead SB, editor. Dengue. Imperial College Press, London, U.K.

Halstead, S.B. 2008. Dengue virus-mosquito interactions. Annu. Rev. Entomol. **53:**273-291.

Halstead, S.B. 2007. Dengue. Lancet. **370:**1644-1652.

Halstead, S.B., S. Rojanasuphot, and N. Sangkawibha. 1983. Original antigenic sin in dengue. Am. J. Trop. Med. Hyg. **32(1):**154-156.

Halstead, S.B. and E.J. O'Rourke. 1977. Dengue viruses and mononuclear phagocytes: I. infection enhancement by non-neutralizing antibody. J. Exper. Med. **146:**201-217.

Halstead, S.B. 1976. Observations related to pathogenesis of dengue hemorrhagic fever. VI. Hypothesis and discussion. Yale J. Bio. and Med. **42:**350-362.

Harris, A.F., A.R. McKemey, D. Nimmo, Z. Curtis, I. Black, S.A. Morgan, M.N. Oviedo, R. Lacroix, N. Nash, N.I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C.A. Donnelly, W.D. Petrie, and L. Alphey 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature Biotech. **30(9):**828-830.

Harris, A.F., D. Nimmo, A.R. McKemey, N. Kelly, S. Scaife, C.A. Donnelly, C. Beech, W.D. Petrie, and L. Alphey. 2011. Field performance of engineered male mosquitoes. Nature Biotech. **29(11):**1034-1039. doi:10.1038/nbt.2019.

Hay, S. 2013. Football fever could be a dose of dengue. Nature. **503:**439.

Hayden, M.H., C.K. Uejio, K. Walker, F. Ramberg, R. Moreno, C. Rosales, M. Gameros, L.O. Mearns, E. Zielinski-Gutierrez, and C.R. Janes. 2010. Microclimate and human factors in the divergent ecology of Aedes aegypti along the Arizona, U.S./Sonora, MX border. EcoHealth. **7:**64-77.

Heberlein-Larson, L., V. Mock, P. Colarusso, S. York, J. Ambrose, A. Cannons, S. Crowe, M.C. Rowlinson, S. Moody-Geissler, and K. Peck. 2014. Outbreak of locally-acquired dengue virus in Florida. [online]. 30th Annual Clinical Virology Symposium and Annual Meeting of the Pan-American Society for Clinical Virology. American Society for Microbiology, Washington, D.C. and The France Foundation, Old Lyme, CT.

Helt, A.M. and E. Harris. 2005. S-phase-dependent enhancement of dengue virus 2 replication in mosquito cells, but not in human cells. J. Virol. **79(21):**13218-13230.

Henderson, B.R., B.J. Saeedi, G. Campagnola, and B.J. Geiss. 2011. Analysis of RNA binding by the dengue virus NS5 RNA capping enzyme. PLoS ONE. **6(10).** doi:10.1371/journal.pone.0025795.

Henson, P.M., and D.L. Bratton. 2009. Recognition and removal of apoptotic cells, p. 341-365. In D.G. Russell and S. Gordon (ed.), Phagocyte-pathogen interactions: macrophages and the host response to infection. ASM Press, Washington D.C.

Hirsch, A. 1883. Dengue, a comparatively new disease: it's symptoms. Handbook of Geographical and Historical Pathology. **1:**55-81.

Hoch, A.L., T.P. Gargan 2nd, and C.L. Bailey. 1985. Mechanical transmission of Rift Valley fever virus by hematophagous Diptera. Am. J. Trop. Med. Hyg. **34(1):**188-193.

Honda, S., M. Saito, E.M. Dimaano, P.A. Morales, M.T.G. Alonzo, L.A.C. Suarez, N. Koike, S. Inoue, A. Kumatori, R.R. Matias, F.F. Natividad, and K. Oishi. 2009. Increased phagocytosis of platelets from patients with secondary dengue virus infection by human macrophages. Am. J. Trop. Med. Hyg. **80(5):**841-845.

Horlbeck, H.B. 1896. Dengue. Public Health Pap. Rep. **22:**191-196.

Hu, D.M., B. Di, X.X. Ding, Y.D. Wang, Y. Chen, Y.X Pan, K. Wen, M. Wang, and X.Y. Che. 2011. Kinetics of non-structural protein 1, IgM, IgG antibodies in dengue type 1 primary infection. Virol. J. (ahead of print). **8(47).** doi:10.1186/1743-422X-8-57.

Huang, K.J., Y.C. Yang, Y.S. Lin, J.H. Huang, H.S. Liu, T.M. Yeh, S.H. Chen, C.C. Liu, and H.Y. Lei. 2006. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. J. Immunol. **176:**2825-2832.

Huang, Y.H., H.Y. Lei, H.S. Liu, Y.S. Lin, C.C. Liu, and T.M. Yeh. 2000. Dengue virus infects human endothelial cells and induces IL-6 and IL-8 production. Am. J. Trop. Med. Hyg. **63(1,2):**71-75.

International Committee on Taxonomy of Viruses (ICTV). 2014. Flavivirdae. http://www.ictvonline.org/virusTaxonomy.asp. (5/4/14)

Imrie, A., J. Meeks, A. Gurary, M. Suhkbaatar, T.T. Truong, C.B. Cropp, and P. Effler. 2007. Antibody to dengue 1 detected more than 60 years after infection. Viral Immunol. **20(4):**672-675.

Jain, M., L. Ganju, A. Katiyal, Y. Padwad, K.P. Mishra, S. Chanda, D. Karan, K.M.S. Yogendra, and R.C. Sawhney. 2008. Effect of Hippophae rhamnoides leaf extract against dengue virus infection in human-derived macrophages. Phytomed. **15:**793-799.

Jessie, K., M.Y. Fong, S. Devi, S.K. Lam, and K.T. Wong. 2004. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. JID. **189:**1411-1418.

Jiang, D., J.M. Weidner, M. Qing, X.B. Pan, H. Guo, C. Xu, X. Zhang, A. Birk, J. Chang, P.Y. Shi, T.M. Block, and J.T. Guo. 2010. Identification of five interferon-induced cellular proteins that inhibit West Nile virus and dengue virus infections. J. Virol. **84(16):**8332-8341.

Johnson, A.J., A.J. Noga, O. Kosoy, R.S. Lanciotti, A.A. Johnson, and B.J. Biggerstaff. 2005. Duplex microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin M antibodies. Clin. Diagn. Lab. Immunol. **12(5):**566-574.

Johnson, A.J., R.C. Cheshier, G. Cosentino, H.P. Masri, V. Mock, R. Oesterle, R.S. Lanciotti, D.A. Martin, A.J. Panella, O. Kosoy, and B.J. Biggerstaff. 2007. Validation of a microsphere-based immunoassay for detection of anti-West Nile virus and anti-St. Louis encephalitis virus immunoglobulin M antibodies. **14(9):**1084-1093.

Johnson, B.W., B.J. Russell, and R.S. Lanciotti. 2005. Serotype-specific detection of dengue viruses in a four-plex real-time reverse transcriptase PCR assay. J. Clin. Microbiol. **43(10):**4977-4983.

Johnson, D., M. Viray, J. Ushiroda, A.C. Whelen, R. Sciulli, R. Gose, R. Lee, E. Honda, and S.Y. Park. 2016. Outbreak of locally-acquired case of dengue fever – Hawaii, 2015. MMWR. **65(2):**34-35.

Kadhiravan, T., A. Saxena, A. Singh, S. Broor, S.K. Sharma, and D.K. Mitra. 2010. Association of intracellular T_H1-T_H2 balance in CD4+ T-cells and MIP-1α in CD8+ T-cells with disease severity in adults with dengue. Imm. Net. **10(5):**164-172.

Kaiser, F., and A. O'Garra. 2009. Cytokines and macrophages and dendritic cells: key modulators of immune responses, p. 281-299. In D.G. Russell and S. Gordon (ed.), Phagocyte-pathogen interactions: macrophages and the host response to infection. ASM Press, Washington D.C.

Kanagaraj, A.P., Verma, D., and Daniell, H. 2011. Expression of dengue-3 premembrane and envelope polyprotein in lettuce chloroplasts. Plant Mol. Biol. **76(3-5):**323-333.

Kanthong, N., C. Laosutthipong, and T.W. Flegel. 2010. Response to dengue virus infections altered by cytokine-like substances from mosquito cell cultures. BMC Microbiol. (ahead of print). **10(290):**doi:10.1186/1471-2180-10-290.

Kaplan, D. and R.D. Schreiber. 1999. The interferons: biochemistry and biology, p. 111-124. In J. Theze (ed.), The cytokine network and immune functions. Oxford University Press, Oxford, U.K.

Kawaguchi, I., A. Sasaki, and M. Boots. 2003. Why are dengue virus serotypes so distantly related? Enhancement and limiting serotype similarity between dengue virus strains. Proc. R. Soc. Lond. **270:**2241-2247.

Kellner, A.W.A. 2008. Global climate change and dengue. An. Acad. Bras. Cienc. **80(2):**215.

Khadka, S., A.D. Vangeloff, C. Zhang, P. Siddavatam, N.S. Heaton, L. Wang, R. Sengupta, S. Sahasrabudhe, G. Randall, M. Gribskov, R.J. Kuhn, R. Perera, and D.J. LaCount. 2011. A physical interaction network of dengue virus and human proteins. Mol. Cell. Proteomics. **10(12)**:doi:10.1074/mcp.M111.012187.

Khan, A.M., O. Miotto, J.M. Nascimento, K.N. Srinivasan, A.T. Heiny, G.L. Zhang, E.T. Marques, T.W. Tan, V. Brusic, J. Salmon, and J.T. August. 2008. Conservation and variability of dengue virus proteins: implications for vaccine design. PLoS Neg. Trop. Dis. **2(8):**e272.

Kleiner, G., A. Marcuzzi, V. Zanin, L. Monasta, and G. Zauli. 2013. Cytokine levels in the serum of healthy subjects. Mediators of Inflammation. **2013:**dx.doi.org/10.1155/2013/434010.

Komar, N. 2003. West Nile virus: epidemiology and ecology in North America, p.185-234. In T.J. Chambers and T.P. Monath (ed.), The flaviviruses: detection, diagnosis and vaccine development. Elsevier Academic Press, London, U.K.

Kontny, U., I. Kurane, and F.A. Ennis. 1988. Gamma interferon augments Fc_{γ} receptor-mediated dengue virus infection of human monocytic cells. J. Virol. **62(11):**3928-3933.

Kopp, A., T.R. Gillespie, D. Hobelsberger, A. Estrada, J.M. Harper, R.A. Miller, I. Eckerle, M.A. Muller, L. Podsladowski, F.H. Leendertz, C. Drosten, and S. Junglen. 2013. Provenance and geographic spread of St. Louis encephalitis virus. mBio. **4(3):**doi:10.1128/mBio.00322-13.

Koraka, P., C.P. Burghoorn-Maas, A. Falconar, T.E. Setiati, K. Djamiatun, J. Groen, and A.D.M.E. Osterhaus. 2003. Detection of immune-complexed-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. J. Clin. Microbiol. **41(9):**4154-4159. doi:10.1128/JCM.41.9.4154-4159.2003.

Kramer, L.D. and L.J. Chandler. 2001. Phylogenetic analysis of the envelope gene of St. Louis encephalitis virus. Arch. Virol. **146:**2341-2355.

Kuadkitkan, A., N. Wikan, C. Fongsaran, D.R. Smith. 2010. Identification and characterization of prohibitin as a receptor protein mediating DENV-2 entry into insect cells. Virology. **doi:10.1016/j.virol.2010.07.015.**

Kularatne, S.A.M., M.M.K. Pathirage, and S. Gunasena. 2008. A case series of dengue fever with altered consciousness and electroencephalogram changes in Sri Lanka. Trans. Roy. Soc. Trop. Med. Hyg. **102:**1053-1054.

Kumar, M., S. Verma, and V.R. Nerurkar. 2010. Pro-inflammatory cytokines derived from West Nile virus (WNV)-infected SK-N-SH cells mediate neuroinflammatory markers and neuronal death. J. Neuroinflamm. **7(73).**

Kuno, G. 2009. Emergence of the severe syndrome and mortality associated with dengue and dengue-like illness: historical records (1890 to 1950) and their compatibility with current hypotheses on the shift of disease manifestation. Clin. Microbiol. Rev. **22(2):**186-201.

Kyle, J.L. and E. Harris. 2008. Global spread and persistence of dengue. Annu. Rev. Microbiol. **62:**71-92.

Lacroix, R., A.R. McKemey, N. Raduan, L.K. Wee, W.H. Ming, T.G. Ney, S. Rahidah A.A., S. Salman, S. Subramaniam, O. Nordin, N. Hanum A.T., C. Angamuthu, S.M. Mansor, R.S. Lees, N. Naish, S. Scaife, P. Gray, G. Labbe, C. Beech, D. Nimmo, L. Alphey, S.S. Vasan, L.H. Lim, N.Wasi A., and S.

Murad. 2012. Open field release of genetically engineered sterile male Aedes aegypti in Malaysia. PLoS One. **7(8):**e42771. doi:10.1371/journal.pone.0042771.

Ladbury, G.A.F., M. Gavana, K. Danis, A. Papa, D. Papamichail, S. Mourelatos, S. Gewehr, G. Theocharopoulos, S. Bonovas, A. Benos, and T. Panagiotopoulos. 2013. Population seroprevalence study after a West Nile virus lineage 2 epidemic, Greece, 2010. PLoS One. **8(11):**e80432. doi:10.1371/journal.pone.0080432.

Lai, Y.C., Y.C. Chuang, C.C. Liu, T.S. Ho, Y.S. Lin, R. Anderson, and T.M. Yeh. 2017. Antibodies against modified NS1 wing domain peptide protect against dengue virus infection. Sci. Rep. **7(1):**6975. doi: 10.1038/s41598-017-07308-3.

Lambeth, C.R., L.J. White, R.E. Johnston, and A.M. de Silva. 2005. Flow cytometry-based assay for titrating dengue virus. J. Clin. Microbiol. **43(7):**3267-3272.

Lanciotti, R.S., G.D. Ebel, V. Deubel, A.J. Kerst, S. Murri, R. Meyer, M. Bowen, N. McKinney, W.E. Morrill, M.B. Crabtree, L.D. Kramer, and J.T. Roehrig. 2002. Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology. **298:**96-105.

Lanciotti, R.S. and A.J. Kerst. 2001. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. J. Clin. Microbiol. **39(12):**4506-4513.

Lanciotti, R.S., A.J. Kerst, R.S. Nasci, M.S. Godsey, C.J. Mitchell, H.M. Savage, N. Komar, N.A. Panella, B.C. Allen, K.E. Volpe, B.S. Davis, and J.T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by taqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. **38(11):**4066-4071.

Lanciotti, R.S., J.T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K.E. Volpe, M.B. Crabtree, J.H. Scherret, R.A. Hall, J.S. MacKenzie, C.B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H.M. Savage, W. Stone, T. McNamara, and D.J. Gubler. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science. **286:**2333-2337.

Landry, M.L. and K. St. George. 2017. Laboratory diagnosis of Zika virus infection. Arch. Pathol. Lab. Med. **141:**60-67.

Lanciotti, R.S., C.H. Calisher, D.J. Gubler, G.J. Chang, and A.V. Vorndam. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbiol. **30(3):**545-551.

Laoprasopwattana, K., D.H. Libraty, T.P. Endy, A. Nisalak, S. Chunsuttiwat, D.W. Vaughn, G. Reed, F.A. Ennis, A.L. Rothman, and S. Green. 2005. Dengue virus (DV) enhancing antibody activity in preillness plasma does not predict subsequent disease severity or viremia in secondary DV infection. JID. **192:**510-519, 1863 (erratum).

Lapphra, K., A. Sangcharaswichai, K. Chokephaibulkit, S. Tiengrim, W. Piriyakarnsakul, T. Chakorn, S. Yoksan, L. Wattanamongkosil, and V. Thamlikitkul. 2008. Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. Diag. Microbiol. Inf. Dis. **60:**387-391.

Larrick, J.W., D.G. Fischer, S.J. Anderson, and H.S. Koren. 1980. Characterization of a human macrophage-like cell line stimulated *in vitro*: a model of macrophage functions. **125(1):**6-12.

Lazear, H.M., A.K. Pinto, M.R. Vogt, M. Gale Jr., and M.S. Diamond. 2011. Beta interferon controls West Nile virus infection and pathogenesis in mice. J. Virol. **85(14):**7186-7194.

Lebani, K., M.L. Jones, D. Watterson, A. Ranzoni, R.J. Traves, P.R. Young, and S.M. Mahler. 2017. Isolation of serotype-specific antibodies against dengue virus non-structural protein 1 using phage display and application in a multiplexed serotyping assay. PLoS One. **http://doi.org/10.1371/journal.pone.0180669.**

Lee, W.C., K.Y. Lien, G.B. Lee, and H.Y. Lei. 2008. An integrated microfluidic system using magnetic beads for virus detection. Diag. Microbiol. Inf. Dis. **60:**51-58.

Lee, Y.R., M.T. Liu, H.Y. Lei, C.C. Liu, J.M. Wu, Y.C. Tung, Y.S. Lin, T.M. Yeh, S.H. Chen, and H.S. Liu. 2006. MCP-1, a highly expressed chemokine in dengue haemorrhagic fever/dengue shock syndrome patients, may cause permeability change, possibly through reduced tight junctions of vascular endothelium cells. J. Gen. Virol. **87:**3623-3630.

Lei, H.Y., T.M. Yeh, H.S. Liu, Y.S. Lin, S.H. Chen, and C.C. Liu. 2001. Immunopathogenesis of dengue virus infection. J. Biomed. Sci. **8:**377-388.

Leparc-Goffart, I., M. Barragatti, S. Temmam, A. Tuiskunen, G. Moreau, R. Charrel, and X. de Lamballerie. 2009. Development and validation of real-time one-step reverse transcription-PCR for the detection and typing of dengue viruses. J. Clin. Virol. **45(1):**61-66.

Leroy, E.M., D. Nkoghe, B. Ollomo, C. Nze-Nkogue, P. Becquart, G. Grard, X. Pourrut, R. Charrel, G. Moureau, A. Ndjoyi-Mbiguino, and X. De Lamballerie. 2007. Concurrent chikungunya and dengue virus infections during simultaneous outbreaks, Gabon. Emerging Infectious Diseases. **15(4):**591-593.

Li, D., W.B. Lott, K. Lowry, A. Jones, H.M. Thu, and J. Aaskov. 2011. Defective interfering particles in acute dengue infections. PLoS ONE. **6(4)** doi:10.1371/journal.pone.0019447.

Li, Q., S. MacDonald, C. Bienek, P.R. Foster, and A.J. Macleod. 2005. Design of a UV-C irradiation process for the inactivation of viruses in protein solutions. Biologicals. **33:**101-110.

Li, Y.Z., D. Counor, P. Lu, G.D. Liang, T.H.Q. Vu, T.N. Phan, T.K.L. Huynh, G. Sun, M. Grandadam, S. Butrapet, J.P. Lavergne, M. Flamand, Y.X. Yu, T. Solomon, P. Buchy, and V. Deubel. 2012. A specific and sensitive antigen capture assay for NS1 protein quantitation in Japanese encephalitis virus infection. J. Virol. Met. **179:**8-16.

Libraty, D.H., L.P. Acosta, V. Tallo, E. Segubre-Mercado, A. Bautista, J.A. Potts., R.G. Jarman, I.K. Yoon, R.V. Gibbons, J.D. Brion, and R.Z. Capeding. 2009. A prospective nested case-control study of dengue in infants: rethinking and refining the antibody-dependent enhancement dengue hemmorhagic fever model. PLoS Medicine. **6(10):**e1000171.

Libraty D.H., P.R. Young, D. Pickering, T.P. Endy, S. Kalayanarooj, S. Green, D.W. Vaughn, A. Nisalak, F.A. Ennis, A.L. Rothman. 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J. Infect. Dis. **186(8):**1165-8.

Likos, A., I. Griffin., A.M. Bingham, D. Stanek, M. Fischer, S. White, J. Hamilton, L. Eisenstein, D. Atrubin, P. Mula, B. Scott, P. Jenkins, D. Fernandez, E. Rico, L. Gillis, R. Jean, M. Cone, C. Blackmore, J. McAllister, C. Vasquez, L. Rivera, C. Philip. 2016. Local mosquito-borne transmission of Zika virus – Miami-Dade and Broward Counties, Florida, June – August 2016. MMWR. **65(38):**1032-1038. doi:10.15585/mmwr.mm6538e1.

Lim, J.K., and P.M. Murphy. 2011. Chemokine control of West Nile virus infection. Exp. Cell. Res. **317:**569-574.

Lin, C.F., S.C. Chiu, Y.L. Hsiao, S.W. Wan, H.Y. Lei, A.L. Shiau, H.S. Liu, T.M. Yeh, S.H. Chen, C.C. Liu, and Y.S. Lin. 2005. Expression of cytokine, chemokines, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. J. Immunol. **175:**395-403.

Lin, C.F., H.Y. Lei, A.L. Shiau, C.C. Liu, H.S. Liu, T.M. Yeh, S.H. Chen, S.C. Chiu, and Y.S. Lin. 2003. Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. J. Med. Virol. **69:**82-90.

Lin, C.F., H.Y. Lei, A.L. Shiau, H.S. Liu, T.M. Yeh, S.H. Chen, C.C. Liu, S.C. Chiu, and Y.S. Lin. 2002. Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. J. Immunol. **169:**657-664.

Lin, S.W., Y.C. Chuang, Y.S. Lin, H.Y. Lei, H.S. Liu, and T.M. Yeh. 2011. Dengue virus nonstructural protein NS1 binds to prothrombin/thrombin and inhibits prothrombin activation. J. Inf. **doi:10.1016/j.jinf.2011.11.023.**

Lin, Y.S., T.M. Yeh, C.F. Lin, S.W. Wan, Y.C. Chuang, T.K. Hsu, H.S. Liu, C.C. Liu, R. Anderson, and H.Y. Lei. 2011. Molecular mimicry between virus and host and its implications for dengue disease pathogenesis. Exp. Biol. Med. **236:**515-523.

Liu, C.C. and S.C. Wu. 2004. Mosquito and mammalian cells grown on microcarriers for four-serotype dengue virus production: variations in virus titer, plaque morphology, and replication rate. Wiley InterScience. **doi:10.1002/bit.10918.**

Liu, I.J., C.Y. Chiu, Y.C. Chen, and H.C. Wu. 2011. Molecular mimicry of human endothelial cell antigen by autoantibodies to nonstructural protein 1 of dengue virus. J. Biolog. Chem. **286(11):**9726-9736. doi:10.1074/jbc.M110.170993.

Lorenz C.M., B.M. Wolk, C.P. Quan, E.W. Alcala, M. Eng, D.J. McDonald, and T.C. Matthews. 2009. The effect of low intensity ultraviolet-C light on monoclonal antibodies. Biotechnology Progress. doi:10.1002/btpr.157

Lowe, R., C. Barcellos, C.A.S. Coelho, T.C. Bailey, G.E. Coelho, R. Graham, T. Jupp, W.M. Ramalho, M. Sa Carvalho, D.B. Stephenson, and X. Rodo. 2014. Dengue outlook for the World Cup in Brazil: an early warning model framework driven by real-time seasonal climate forecasts. Lancet Infect. Dis. **14(707):**81-89.

Macdonald, J., J. Tonry, R.A. Hall, B. Williams, G. Palacios, M.S. Ashok, O. Jabado, D. Clark, R.B. Tesh, T. Briese, and W.I. Lipkin. 2005. NS1 protein secretion during the acute phase of West Nile infection. J. Virol. **79(22):**13924-13933.

Mackenzie, J.M., M.K. Jones, and P.R. Young. 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology. **220:**232-240.

Mangada, M.M., and A.L. Rothman. 2005. Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. J. Immunol. **159(4):**2676-2683.

Marques, Jr. 2009. Gene expression profiling during early acute febrile stage of dengue infection can predict the disease outcome. PLoS ONE. **4(11):**e7892.

Marr, J.S. and J.T. Cathey. The 1802 Saint-Domingue yellow fever epidemic and the Louisiana Purchase. J. Public Health Management Practice. **19(1):**77-82.

Marr, J.S. and C.H. Calisher. 2003. Alexander the Great and West Nile virus encephalitis. Emerging Infectious Diseases. **9(12):**1599-1603.

Martina, B.E.E., P. Koraka, and A.D.M.E. Osterhaus. 2009. Dengue virus pathogenesis: an integrated view. Clin. Microbiol. Rev. **22(4):**564-581.

Martins Vargas, H.C., L.C. Farnesi, A.J. Martins, D. Valle, and G.L. Rezende. 2014. Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors Aedes aegypti, Anopheles aquasalis and Culex quinquefasciatus. J. Insect Physiol. **62(2014):**54-60.

Mason, P.W., M.U. Zugel, A.R. Semproni, M.J. Fournier, and T.L. Mason. 1990. The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in Escherichia coli. J. Gen. Virol. **71:**2107-2114.

Mata, V.E., S.R.L. Passos, Y.H.M. Hokerberg, G.M. Berardinelli, M.A.B. dSantos, L.V.B. Fukuoka, A.C.F.S.R. Maciel, C.D.S. Rodrigues, A.S. Santos, and R.V.C. dOliveira. 2017. Precisao e confiabilidade de um teste imuno-cromatografico rapido NS1 para diagnostic DENV-1 no ponto de atendimento e no laboratorio (in English). BMC Infect. Dis. **17(594).** doi:10.1186/s12879-017-2679-z.

Mayuri, E.L.B. and R.J. Kuhn. 2010. Novel therapeutic approaches for dengue disease, p. 239-264. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Meltzer, E., Z. Heyman, H. Bin, and E. Schwartz. 2012. Capillary leakage in travelers with dengue infection: implications for pathogenesis. Am. J. Trop. Med. Hyg. **86(3):**536-539.

Metsky, H.C., C.B. Matranga, S. Wohl, S.F. Schaffner, C.A. Freije, S.M. Winnicki, K. West, J. Qu, M.L. Baniecki, A. Gladden-Young, A.E. Lin, CH. Tomkins-Tinch, S.H. Ye, D.J. Park, C.Y. Luo, K.G. Barnes, R.R. Shah, B. Chak, G. Barbosa-Lima, E. Delatorre, Y.R. Viera, L.M. Paul, A.L. Tan, C.M. Barcellona, M.C. Porcelli, C. Vasquez, A.C. Cannons, M.R. Cone, K.N. Hogan, E.W. Kopp IV, J.J. Anzinger, K.F. Garcia, L.A. Parham, R.M.G. Ramirez, M.C.M. Montoya, D.P. Rojas, C.M. Brown, S. Hennigan, B. Sabina, S. Scotland, K. Gangavarapu, N.D. Grubaugh, G. Oliveira, R. Robles-Sikisaka, A. Rambaut, L. Gehrke, S. Smole, M.E. Halloran, L. Villar, S. Mattar, I. Lorenzana, J. Cerbino-Neto, C. Valim, W. Degrave, P.T. Bozza, A. Gnirke, K.G. Andersen, S. Isern, S.F. Michael, F.A. Bozza, T.M.L. Souza, I. Bosch, N.L. Yozwiak, B.L. MacInnis, and P.C. Sabeti. 2017. Zika virus evolution and spread in the Americas. Nature. **546(7658):**411-415. doi:10.1038/nature22402.

Miami Herald. 2016. First Zika, now dengue. New case pops up in Miami area. http://www.miamiherald.com/news/health-care/article104606196.html.

Midgley, C.M., M. Bajwa-Joseph, S. Vasanawathana, W. Limpitikul, B. Wills, A. Flanagan, E. Waiyaiya, H.B. Tran, A.E. Cowper, P. Chotiyarnwon, J.M. Grimes, S. Yoksan, P. Malasit, C.P. Simmons, J. Mongkolsapaya, and G. Screaton. 2011. An in-depth analysis of original antigenic sin in dengue virus infection. J. Virol. **85(1):**410-421.

Miller, S., I. Romero-Brey, and R. Bartenschlager. 2010. The dengue virus replication complex, p.35-53. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Mondini A., R.V. de Morais Bronzoni, I.L. Soares Cardeal, T.M.I. Lopes dos Santos, E. Lazaro, S.H. Pereira Nunes, G.C. Dutra Silva, M.C. Ferrari Sarkis Madrid, P. Rahal, L. Tadeu Figueiredo, F. Chiaravalloti Neto, M. Lacerda Nogueira. 2007. Simultaneous infection by DENV-3 and SLEV in Brazil. J Clin Virol. **40:**84-86.

Moore, Jr., F. 2004. Complement, p.85-109. In G.B. Pier, J.B. Lyczak, and L.M. Wetzler (ed.). Immunology, infection, and immunity. ASM Press. Washington, D.C.

Moreira, L.A., I. Iturbe-Ormaetxe, J.A. Jeffrey, G. Lu, A.T. Pike, L.M. Hedges, B.C. Rocha, S. Hall-Mendelin, A. Day, M. Riegler, L.E. Hugo, K.N. Johnson, B.H. Kay, E.A. McGraw, A.F. van den Hurk, P.A. Ryan, and S.L. O'Neill. 2009. A Wolbachia symbiont in Aedes aegypti limits infection with dengue, chikungunya, and Plasmodium. Cell. **139:**1268-1278.

Morens, D.M. and A.S. Fauci. 2008. Dengue and hemorrhagic fever: a potential threat to public health in the United States. JAMA. **299(2):**214-216.

Mok, Y., J. Quah, and C. Siau. 2013. A rare but potentially lethal complication of dengue. Asian Pac. J. Trop. Med. **6(6):**500-501.

Mondini, A. and F. Chiaravalloti-Neto. 2008. Spatial correlation of incidence of dengue with socioeconomic, demographic and environmental variables. Sci. Total Env. **393:**241-248.

Mondini, A., R. Vieira de Morais Bronzoni, I.L. Soares Cardeal, T.M. Lopes dos Santos, E. Lazaro, S.H. Pereira Nunes, G.C. Dutra Silva, M.C. Ferrari Sarkis Madrid, P. Rahal, L. Tadeu Figueiredo, F. Chiaravalloti Neto, and M. Lacerda Nogueira. 2007. Simultaneous infection by DENV-3 and SLEV in Brazil. J. Clin. Virol. **40:**84-86.

Moreira, L.A., I. Iturbe-Ormaetxe, J.A. Jeffery, G. Lu, A.T. Pyke, L.M. Hedges, B.C. Rocha, S. Hall-Mendelin, A. Day, M. Riegler, L.E. Hugo, K.N. Johnson, B.H. Kay, E.A. McGraw, A.F. van den Hurk, P.A. Ryan, and S.L. O'Neill. 2009. A Wolbachia symbiont in Aedes aegypti limits infection with dengue, chikungunya, and Plasmodium. Cell. **139(7):**1268-1278.

Morens, D.M. and A.S. Fauci. 2008. Dengue and hemorrhagic fever: a potential threat to public health in the United States. JAMA. **299(2):**214-216.

Morens, D.M., G.K. Folkers, and A.S. Fauci. 2013. Dengue: the continual re-emergence of a centuries-old disease. EcoHealth. **doi:10.1007/s10393-013-0825-7.**

Mosquera, J.A., Hernandez, J.P., Valero, N., Espina, L.M., and Anez, G,J. 2005. Ultrastructural studies on dengue virus type 2 infection of cultured human monocytes. Virol. J. **2(26):**doi:10.1186/1743-422X-2-26.

Mosso, C., I.J. Galvan-Mendoza, J.E. Ludert, and R.M. del Angel. 2008. Endocytic pathway followed by dengue virus to infect the mosquito cell line C6/36 HT. Virology. **378(1):**193-199.

Muller, D.A., M.J. Landsberg, C. Bletchly, R. Rothnagel, L. Waddington, B. Hankamer, and P.R. Young. 2012. Structure of the dengue virus glycoprotein non-structural protein 1 by electron microscopy and single-particle analysis. J. Gen. Virol. **93:**771-779.

Munoz-Jordan, J.L., G.A. Santiago, H. Margolis, and L. Stark. 2013. Genetic relatedness of dengue viruses in Key West, Florida, USA, 2009-2012. EID. **19(4):**652-654.

Munoz-Jordan, J.L. and I. Bosch. 2010. Modulation of the antiviral response by dengue virus, p. 121-140. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Nascimento, E.J.M., U. Braga-Neto, C.E. Calzavara-Silva, A.L.V. Gomes, F.G.C. Abath, C.A.A. Brito, M.T. Cordeiro, A.M. Silva, C. Magalhaes, R. Andrade, L.H.V.G. Gil, and E.T.A. 2009. Gene expression profiling during early acute febrile stage of dengue infection can predict the disease outcome. PLoS One. **4(11):**e7892. doi:10.1371/journal.pone.0007892.

Nasirudeen, A.M.A., H.H. Wong, P. Thien, S. Xu, K.P. Lam, and D.X. Liu. 2011. RIG-1, MDA5, and TLR3 synergystically play an important role in restriction of dengue virus infection. PLoS Negl Trop Dis. **5(1):**e926. doi:10.1371/journal.pntd.0000926.

Nawa, M., T. Takasaki, T., M. Ito, S. Inoue, K. Morita, and I. Kurane. 2005. Immunoglobulin A antibody responses in dengue patients: a useful marker for serodiagnosis of dengue virus infection. Clin. Diagn. Lab. Immunol. **12(10):**1235-1237.

National Center for Biotechnology Information (NCBI). 2014. Flaviviridae. (5/4/14) http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=11050&lvl=3&lin=f&keep=1&srchmode=1&unlock.

Newton-Nash, D.K. and P.J. Newman. 1999. A new role for platelet-endothelial cell adhesion molecule-1 (CD31): inhibition of TCR-mediated signal transduction. J. Immunol. **163(2):**682-688.

Nevai, A.L. and E. Soewono. 2013. A model for the spatial transmission of denue with daily movement between villages and a city. Math. Med. Biol. (ahead of print). **doi:10.1093/imammb/dqt002.**

NI Reis, S.R., A.L.F. Sampaio, M. das Gracas Muller Henriques, M. Gandini, E. Leal Azeredo, and C. Fernandes Kubelka. 2007. An *in vitro* model for dengue virus infection that exhibits human monocyte infection, multiple cytokine production and dexamethasone immunomodulation. Mem. Inst. Oswaldo Cruz. **102(8):**983-990.

Nicod, L.P. and J.M. Dayer. 1999. Cytokines in the functions of dendritic cells, monocytes, macrophages and fibroblasts, p. 210-220. In J. Theze (ed.), The cytokine network and immune functions. Oxford University Press, Oxford, U.K.

Nimmannitya, S. 1997. Dengue hemorrhagic fever: diagnosis and management, p.133-146. In D.J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International.

Nisalak, A., W.H. Bancroft, R. McNair Scott, S. Vithanomsat, and N. Nutkumhang. 1976. Detection of dengue infected mosquitoes by direct fluorescent antibody microscopy. AFRIMS. http://www.afrims.org/weblib/eapr/1976/APR76p011-015.pdf.

Nishiura, H. and S.B. Halstead. 2007. Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. J. Infect. Dis. **195:**1007-1013.

Noisekaran, S., N. Onlamoon, H.M. Hsiao, K.B. Clark, F. Villinger, A.A. Ansari, and G.C. Perng. 2012. Infection of bone marrow cells by dengue virus *in vivo*. Exp. Hematol. **40(3):**250-259.e4.

Noisakran, S., T. Dechtawewat, P. Rinkaewkan, C. Puttikhunt, A. Kanjanahaluethai, W. Kasinrerk, N. Sittisombut, and P. Malasit. 2007. Characterization of dengue virus NS1 stably expressed in 293T cell lines. J. Virol. Meth. **142:**67-80.

Nobuchi, H. 1979. The symptoms of a dengue-like illness recorded in a Chinese medical encyclopedia (Japanese). Kanpo no Rinsho. **26:**422-425.

NPR. 2016. Florida Keys approves trial of genetically modified mosquitoes to fight Zika. http://www.npr.org/sections/health-shots/2016/11/20/502717253/florida-keys-approves-trial-of-genetically-modified-mosquitoes-to-fight-zika.

Ochoa, M.V., J.G. Cordero, B.G. Castaneda, L.S. Argumedo, N.V. Sepulveda, and L.C. Barron. 2009. A clinical isolate of dengue virus and its protens induce apoptosis in HMEC-1 cells: a possible implication in pathogenesis. **154:**919-928. doi:10.1007/s00705-009-0396-7.

Ong, S.P., L.M. Lee, Y.F.I. Leong, M.L. Ng, J.J.H. Chu. 2012. Dengue virus infection mediates HMGB1 release from monocytes involving PCAF acetylase complex and induces vascular leakage in endothelial cells. PLoS ONE. 7**(7):**e41932.

Ooi, E.E. and D.J. Gubler. 2010. Mosquito-virus interactions, p. 143-155. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Otake, S., S.A. Dee, K.D. Rossow, R.D. Moon, and C. Pijoan. 2002. Mechanical transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, Aedes vexans (Meigen). Can. J. Vet. Res. **66(3):**191-195.

Ottendorfer, C.L., J.H. Ambrose, G.S. White, T.R. Unnasch, and L.M. Stark. 2009. Genotype V St. Louis encephalitis virus isolated in Florida. Emerg. Infect. Dis. **15(4):**604-606.

Ottendorfer, C.L. 2008. Impact of West Nile virus on the natural history of St. Louis encephalitis virus in Florida. Ph.D. dissertation. University of South Florida.

Paddock, C.D., W.L. Nicholson, J. Bhatnagar, C.S. Goldsmith, P.W. Greer, E.B. Hayes, J.A. Risko, C. Henderson, C.G. Blackmore, R.S. Lanciotti, G.L. Campbell, and S.R. Zaki. 2006. Fatal hemorrhagic fever caused by West Nile virus in the United States. CID. **42:**1527-1535.

Padmanabha, H., E. Soto, M. Mosquera, C.C. Lord, and L.P. Lounibos. 2010. Ecological links between water storage behaviors and Aedes aegypti production: implications for dengue vector control in variable climates. EcoHealth. **7:**78-90.

Paingankar, M.S., M.D. Gokhale, and D.N. Deobagkar. 2010. Dengue-2-virus-interacting polypeptides involved in mosquito cell infection. Arch. Virol. **155:**1453-1461.

Palmer, C.J., S.D. King, R.R. Cuadrado, E. Perez, M. Baum, and A.L. Ager. 1999. Evaluation of the MRL diagnostics dengue fever virus IgM capture ELISA and the PanBio rapid immunochromatographic test for diagnosis of dengue fever in Jamaica. J. Clin. Microbiol. **37(5):**1600-1601.

Pan-American Health Organization (PAHO). 2014. Description of the current epidemiological trends of dengue in the Americas.
http://www.paho.org/hq/index.php?option=com_content&view=article&id=4494&Itemid=2481&lang=en

Pan-American Health Organization (PAHO). 2011. Number of reported cases of dengue hemorrhagic fever (DHF) in the Americas, by country: 2008
http://ais.paho.org/atlas/dengue/paneldengue1.html

Pan-American Health Organization (PAHO). 2011. The history of dengue and dengue hemorrhagic fever (DHF) in the region of the Americas, 1635–2001.
http://www.paho.org/English/AD/DPC/CD/dengue_history.htm

Pan-American Health Organization (PAHO). 1997. Hemispheric plan to expand and intensify efforts to combat Aedes aegypti. Caracas, Venezuela.

Pang, T., M.J. Cardosa, and M.G. Guzman. 2007. Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). Immunology and Cell Biology. **85:**43-45.

Panyasrivanit, M., M.P. Greenwood, D. Murphy, C. Isidoro, P. Auewarakul, and D.R. Smith. 2011. Induced autophagy reduces virus output in dengue infected monocytic cells. Virology. **418:**74-84. doi:10.1016/j.virol.2011.07.010.

Parameswaran, N. and S. Patial. 2010. Tumor necrosis factor-α signaling in macrophages. Crit. Rev. Eukaryot. Gene Expr. **20(2):**87-103.

Parquet, M.C., A. Kumatori, F. Hasebe, E.G.M. Mathenge, and K. Morita. 2002. St. Louis encephalitis virus induced pathology in cultured cells. Arch. Virol. **147:**1105-1119.

Pattanakitsakul, S.N., J. Poungsawai, R. Kanlaya, S. Sinchaikul, S.T. Chen, and V. Thongboonkerd. 2010. Association of Alix with late endosomal lysobisphosphatidic acid is important for dengue virus infection in human endothelial cells. J. Proteome Res. **9:**4640-4648.

Patterson, G. 2004. 9. The mosquito wars: pesticides, impoundments, and mosquito control, p.152-174. In The mosquito wars: a history of mosquito control in Florida. University Press of Florida, Gainesville, Florida.

Paupy, C., H. Delatte, L. Bagny, V. Corbel, and D. Fontenille. 2009. Aedes albopictus, an arbovirus vector: from the darkness to the light. Mic. Inf. **11:**1177-1185.

Perera, R., M. Khaliq, and R.J. Kuhn. 2008. Closing the door on flaviviruses: entry as a target for antiviral drug design. Antivir. Res. **80:**11-22.

Perera, R. and R.J. Kuhn. 2008. Structural proteomics of dengue virus. Curr. Op. Microbiol. **11:**369-377.

Petersen, L.R. and A.D.T. Barrett. 2009. Arthropod-borne flaviviruses, p.1173-1214. In D.D. Richman, R.J. Whitley, and F.G. Hayden (ed.), Clinical Virology, 3rd Ed. ASM Press, Washington D.C.

Peyrefitte, C.N., B. Pastorino, G.E. Grau, J. Lou, H. Tolou, and P. Couissinier-Paris. 2006. Dengue virus infection of human microvascular endothelial cells from different vascular beds promotes both common and specific functional changes. J. Med. Virol. **78**:229-242.

Pierce, J.R. 2003. "In the interest of humanity and the cause of science": the yellow fever volunteers. Military Medicine. **168(11):**857-863.

Poungsawai, J., R. Kanlaya, S.N. Pattanakitsakul, and V. Thongboonkerd. 2011. Subcellular localizations and time-course expression of dengue envelope and non-structural 1 proteins in human endothelial cells. Microbial Pathogenesis. **51:**225-229.

ProMed-Mail. 2014. http://www.promedmail.org/

Pryor, M.J. and P.J. Wright. 1994. Glycosylation mutants of dengue virus NS1 protein. J. Gen. Virol. **75:**1183-1187.

Puerta-Guardo, H., S.I. De la Cruz Hernández, V.H. Rosales, J.E. Ludert, and R.M. del Angel. 2012. The 1α,25-dihydroxy-vitamin D3 reduces dengue virus infection in human myelomonocyte (U937) and hepatic (Huh-7) cell lines and cytokine production in the infected monocytes. Antiviral Res. **94:**57-61.

Puttikhunt, C., T. Prommool, N. U-thainual, P. Ong-ajchaowlerd, K. Yoosook, C. Tawilert, T. Duangchinda, N. Jairangsri, N. Tangthawornchaikul, P. Malasit, and W. Kasinrerk. 2011. The development of a novel serotyping-NS1-ELISA to identify serotypes of dengue virus. J. Clin. Virol. **doi:10.1016/j.jcv.2011.01.001.**

Putvatana, R., S. Yoksan, T. Chayayodhin, N. Bhamarapravati, and S.B. Halstead. 1984. Absence of dengue 2 infection enhancement in human sera containing Japanese encephalitis antibodies. Am. J. Trop. Med. Hyg. **33(2):**288-294.

Qiagen. 2005. QIAamp® viral RNA mini handbook.

Qiagen. 2002. Taq PCR handbook.

Qiu, L.W., B. Di, K. Wen, X.S. Wang, W.H. Liang, Y.D. Wang, Y.X. Pan, M. Wang, Y.Q. Ding, and X.Y. Che. 2009. Development of an antigen capture immunoassay based on monoclonal antibodies specific

for dengue virus serotype 2 nonstructural protein 1 for early and rapid identification of dengue virus serotype 2 infections. Clin. Vaccine Immunol. **16(1):**88-95.

Rajadhyaksha, A. and S. Mehra. 2012. Dengue fever evolving into systemic lupus erythematosus and lupus nephritis. Lupus. **0:**1-4.

Ramasamy, R., S.N. Surendran, P.J. Jude, S. Dharshini, and M. Vinobaba. 2011. Larval development of Aedes aegypti and Aedes albopictus in peri-urban brackish water and its implications for transmission of arboviral diseases. PLoS Neg. Trop. Dis. **5(11):**doi:10.1371/journal.pntd.001369.

Randolph, G.J. 2008. Emigration of monocyte-derived cells to lymph nodes during resolution of inflammation and its failure in atherosclerosis. Curr. Opin. Lipidol. **19:**462-468.

Rappole, J.H., S.R. Derrickson, and Z. Hubalek. 2000. Migratory birds and spread of West Nile virus in the Western Hemisphere. EID. **6(4):**319-328.

Rathakrishnan, A., B. Klekamp, S.M. Wang, T.V. Komarasamy, S.K. Natkunam, J. Sathar, A. Azizan, A. Sanchez-Anguiano, R. Manikam, and S.D. Sekaran. 2014. Clinical and immunological markers of dengue progression in a study cohort from a hyperendemic area in Malaysia. PLoS One. **9(3):**e92021. doi:10.1371/journal.pone.0092021.

Rawlings, J.A., K.A. Hendricks, C.R. Burgess, R.M. Campman, G.G. Clark, L.J. Tabony, and M.A. Patterson. 1998. Dengue surveillance in Texas, 1995. Am. J. Trop. Med. Hyg. **59(1):**95-99.

Reagan, R.L., and A.L. Brueckner. 1952. Studies of dengue fever virus in the cave bat (Myotus lucifugus). J. Inf. Dis. **91(2):**145-146.

Reed, W. 1902. Recent researches concerning the etiology, propagation, and prevention of yellow fever, by the United States Army Comission. J. Hyg. Lond. **2(2):**101-109.

Reed, W., J. Carroll, A. Agramonte, and J.W. Lazear. 1900. The etiology of yellow fever- a preliminary note. Public Health Pap. Rep. **26:**37-53.

Regis, L., S.B. da Silva, and A.V. Melo-Santos. 2000. The use of bacterial larvicides in mosquito and black fly control programmes in Brazil. Mem. Inst. Oswaldo Cruz. **95(1):**207-210.

Reisen, W.K., H.D. Lothrop, S.S. Wheeler, M. Kennsington, A. Gutierrez, Y. Fang, S. Garcia, and B. Lothrop. Persistent West Nile virus transmission and the apparent displacement St. Louis encephalitis virus in southeastern California, 2003-2006. J. Med. Entomol. **45(3):**494-508.

Reisen, W.K. 2003. Epidemiology of St. Louis encephalitis virus, p.139-183. In T.J. Chambers and T.P. Monath (ed.), The flaviviruses: detection, diagnosis and vaccine development. Elsevier Academic Press, London, U.K.

Restrepo, B.N., D.M. Isaza, C.L. Salazar, R. Ramirez, M. Ospina, and L.G. Alvarez. 2008. Serum levels of interleukin-6, tumor necrosis factor-alpha and interferon-gama in infants with and without dengue. Revista da Sociedade Brasileira de Medicina Tropical. **41(1):**6-10.

Rezende, G.L., A.J. Martins, C. Gentile, L.C. Farnesi, M. Pelajo-Machado, A.A. Peixoto, and D. Valle. 2008. Embryonic desiccation resistance in Aedes aegypti: presumptive role of the chitinized serosal cuticle. BMC Dev. Biol. **8(182):**doi:10.1186/1471-213X-8-82.

Roberts, P.L. 2008. Virus inactivation by solvent/detergent treatment using Triton X-100 in a high purity factor VIII. Biologicals. **36:**330-335.

Rodriguez-Madoz, J.R., A. Belicha-Villanueva, D. Bernal-Rubio, J. Ashour, J. Ayllon, and A. Fernandez-Sesma. 2010. Inhibition of the type I interferon response in human dendritic cells by dengue virus infection requires a catalytically active NS2B3 complex. J. Virol. **84(19):**9760-9774.

Roehrig, J.T. 1997. Immunochemistry of dengue viruses, p.199-220. In D.J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International.

Rolph, M.S., A. Zaid, N.E. Rulli, and S. Mahalingam. 2011. Downregulation of interferon-β in antibody-dependent enhancement of dengue viral infections of human macrophages is dependent on interleukin-6. J. Infect. Dis. **204:**489-491.

Rothman, A.L. 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat. Rev. Immunol. **11:**532-543.

Ruhul Amin M., A.H. Mahbub, A.R. Sikder, and M.M. Karim. 2010. Prediction of the post-translational modification sites on dengue virus E protein and deciphering their role in pathogenesis. Int J Bioinform Res Appl. **6(5):**508-21.

Rush, A.B. 1789. An account of the bilious remitting fever, as it appeared in Philadelphia in the summer and autumn of the year 1780. Medical Inquiries and Observations. Prichard and Hall, Philadelphia. 104-117.

Sabin, A.B. 1952. Research on dengue during World War II. Am. J. Trop. Med. Hyg. **1:**30-50.

Sabin, A.B. and I. Young. 1948. A complement fixation test for dengue. Proc. Soc. Exp. Biol. Med. **69(3):**478-480.

Sample, I. Genetically modified mosquitoes lined up to tackle dengue fever. guardian.co.uk. 11 Nov 2010, 19.40 GMT.
http://www.guardian.co.uk/society/2010/nov/11/genetically-modified-mosquitoes-dengue-fever

Samuel, M.A. and M.S. Diamond. 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. J. Virol. **79(21):**13350-13361.

Sanders, M., A. Blumberg, and W. Haymaker. 1953. Polyradiculopathy in man produced by St. Louis encephalitis virus (SLE). South Med. J. **46(6):**606-608.

Scaturro, P., M. Cortese, L. Chatel-Chaix, W. Fischl, and R. Bartenschlager. 2015. Dengue virus nonstructural protein 1 modulates infectious particle production via interaction with the structural proteins. PLoS Pathogens. **11(11):**e1005277. doi:10.1371/journal/ppat.1005277.

Schmitt, M., I.G. Bravo, P.J.F. Snijders, L. Gissmann, M. Pawlita, and T. Waterboer. 2006. Bead-based multiplex genotyping of human papillomaviruses. J. Clin. Microbiol. **44(2):**504-512.

Schlesinger, J.J. 2006. Flavivirus nonstructural protein NS1: complementary surprises. PNAS. **103(50):**18879-18880.

Schneider, E. and M. Dy. 1999. Cytokines and haematopoiesis, p.146-161. In J. Theze (ed.), The cytokine network and immune functions. Oxford University Press, Oxford, U.K.

Schnirring, L. 2013. Reseachers identify fifth dengue subtype. CIDRAP News.
http://www.cidrap.umn.edu/news-perspective/2013/10/researchers-identify-fifth-dengue-subtype

Seligman, S.J. 2008. Constancy and diversity in the flavivirus fusion peptide. Virol J. **5:27.**

Shaman, J., J.F. Day, and M. Stieglitz. 2005. Drought-induced amplification and epidemic transmission of West Nile virus in southern Florida. J. Med. Entomol. **42(2):**134-141.

Shresta, S. 2012. Role of complement in dengue virus infection: protection or pathogenesis. mBio. **3(1):**e00003-12. doi:10.1128/mBio.00003-12.

Shresta, S., K.L. Sharar, D.M. Prigozhin, P.R. Beatty, and E. Harris. 2006. Murine model for dengue virus-induced lethal disease with increased vascular permeability. J. Virol. **80(20):**10208-10217.

Shu, P.Y., S.F. Chang, Y.C. Kuo, Y.Y. Yueh, L.J. Chien, C.L. Sue, T.H. Lin, and J.H. Huang. 2003. Development of group- and serotype-specific one-step SYBR Green I-based real-time reverse transcription-PCR assay for dengue virus. J. Clin. Microbiol. **41(6):**2408-2416.

Sierra, B., A.B. Perez, K. Vogt, G. Garcia, K. Schmotte, E. Aguirre, M. Alvarez, H.D. Volk, and M.G. Guzman. 2010. MCP-1 and MIP-1α expression in a model resembling early immune response to dengue. Cytokine. **doi:10.1016/j.cyto.2010.06.010.**

Silva, B.M., L.P. Sousa, A.C. Gomes-Ruiz, F.G.G. Leite, M.M. Teixeira, F.G. da Fonseca, P.F.P. Pimenta, P.C.P. Ferreira, E.G. Kroon, C.A. Bonjardim. 2011. The dengue virus nonstructural protein 1 (NS1) increases NF-κB transcriptional activity in HepG2 cells. Arch. Virol. **156:**1275-1279.

Simonnet, C., A. Okandze, S. Matheus, F. Djossou, M. Nacher, and A. Mahamat. 2017. Prospective evaluation of the SD BIOLINE Dengue Duo rapid test during a dengue virus epidemic. Eur. J. Clin. Microbiol. Infect. Dis. **doi:10.1007/s10096-017-3083-8.**

Sips, G.J., J. Wilschut, and J.M. Smit. 2012. Neuroinvasive flavivirus infections. Rev. Med. Virol. **22:**69-87.

Smart, W.R.E. 1877. On dengue or dandy fever. Br. Med. J. **1(848):**382-383.

Smith, C.E. 1956. The history of dengue in tropical Asia and its probable relationship to the mosquito Aedes aegypti. J. Trop. Med. Hyg. **59(10):**243-251.

Smith, G.W. and P.J. Wright. 1985. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and *Aedes albopictus* cells. J. Gen. Virol. **66:**559-571.

Solomon, T. and M. Mallewa. 2001. Dengue and other emerging flaviviruses. J Infect. **42**:104-115.

Somnuke, P., R.E. Hauhart, J.P. Atkinson, M.S. Diamond, and P. Avirutnan. 2011. N-linked glycosylation of dengue virus NS1 protein modulates secretion, cell-surface expression, hexamer stability, and interactions with human complement. Virology. **413:**253-264.

Staples, J.E. and T.P. Monath. 2008. Yellow fever: 100 years of discovery. JAMA. **300(8):**960-962.

Suharti, C., E.C.M. van Gorp, T.E. Setiati, W.M.V. Dolmans, R.J. Djokomoeljanto, C.E. Hack, H. ten Cate, J.W.M. van der Meer. 2002. The role of cytokines in activation of coagulation and fibrinolysis in dengue shock syndrome. Thromb. Haemost. **87:**42-46.

Sun, P., S. Fernandez, M.A. Marovich, D.R. Palmer, C.M. Celluzi, K. Boonnak, Z. Liang, H. Subramanian, K.R. Porter, W. Sun, and T.H. Burgess. 2009. Functional characterization of *ex vivo* blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology. **383**:207-215.

Sundstrom and Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer. **17(5):**565-577.

Tan, C.H., P.S.J. Wong, M.Z.I. Li, I. Vythilingam, and L.C. Ng. 2011. Evaluation of the Dengue NS1 Ag Strip® for detection of dengue virus antigen in Aedes aegypti (Diptera: Culicidae). Vect. Zoo. Dis. **11(6):**789-792.

Thayan, R., Z. Saat, and S.D. Sekaran. 2010. Simultaneous detection of dengue specific NS1, IgM, and IgG for the diagnosis of dengue infection. (submitted for publication).

Thermo Scientific, Pierce®. 2011. Nab™ spin columns, 0.2mL for antibody purification, instruction manual. Pierce Biotechnology, Rockford, IL.

Thomas, D.L., G.A. Santiago, R. Abeyta, S. Hinojosa, B. Torres-Velasquez, J.K. Adam, N. Evert, E. Caraballo, E. Hunsperger, J.L. Munoz-Jordan, B. Smith, A. Banicki, K.M. Tomashek, L. Gaul, and T.M. Sharp. 2016. Reemergence of dengue in southern Texas, 2013. EID. **22(6):**1002-1007.

Thomas, J.G. 1880. Dengue. Public Health Pap. Rep. **6:**136-153.

Thomas, S.J., A. Nisalak, K.B. Anderson, D.H. Libraty, S. Kalayanarooj, D.W. Vaughn, R. Putnak, R.V. Gibbons, R. Jarman, and T.P. Endy. 2009. Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: how alterations in assay conditions impact performance. Am. J. Trop. Med. Hyg. **81(5):**825-833.

Tricou, V., N.N. Minh, J. Farrar, H.T. Tran, C.P. Simmons. 2011. Kinetics of viremia and NS1 antigenemia are shaped by immune status and virus serotype in adults with dengue. PLoS Neg. Trop. Dis. **5(9)** doi:10.1371/journal.pntd.0001309.

Tzianabos, A.O. and L.M. Wetzler. 2004. Cellular communication, p. 343-369. In G.B. Pier, J.B. Lyczak, and L.M. Wetzler (ed.). Immunology, infection, and immunity. ASM Press. Washington, D.C.

Unger, R.E., V. Krump-Konvalinkova, K. Peters, and C.J. Kirkpatrick. 2002. *In vitro* expression of the endothelial phenotype: comparative study of primary isolated cells and cell lines, including novel cell line HPMEC-ST1.6R. Microvascular Res. **64:**384-397.

United Nations (UN). 2004. World population to 2300. United Nations, New York, NY.
United States National Institutes of Health (USNIH). 2012. Dengue virus NS1 antigen (Bio-Rad) clinical protocol. http://clinicaltrials.gov/ct2/show/NCT01226173.

Valerio, L., X. de Balanzo, O. Jiminez, M.L. Pedro-Botet. 2006. Haemorrhagic exanthema due to dengue virus induced by acetylsalicylic acid (*in Spanish, Abstract in English)*. An. Sist. Sanit. Navar. **29(3):**439-442.

Van den Herik-Oudijk, *I.E.*, P.J.A. Capel, T. van der Bruggen, and J.G.J. Van de Winkel. 1995. Identification of signaling motifs within human FcγRIIa and FcγRIIb isoforms. Blood. **85(8):**2202-2211.

Vasilakis, N., J. Cardosa, K.A. Hanley, E.C. Holmes, and S.C. Weaver. 2011. Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. Nature Rev. **9:**532-541.

Vasilakis, N., K.A. Hanley, and S.C. Weaver. 2010. Dengue virus emergence from its sylvatic cycle, p. 183-217. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Vasilakis, N. and S.C. Weaver. 2008. The history and evolution of human dengue emergence. Adv. Virus Res. **72** doi:10.1016/S0065-3527(08)00401-6.

Venter, M., F.J. Burt, L. Blumberg, H. Fickl, J. Paweska, and R. Swanepoel. 2009. Cytokine induction after laboratory-aquired West Nile virus infection. New Eng. J. Med. **360(12):**1260-1262.

Vinh, D.C., and S.M. Holland. 2009. Macrophage classical activation, p. 301-323. In D.G. Russell and S. Gordon (ed.), Phagocyte-pathogen interactions: macrophages and the host response to infection. ASM Press, Washington D.C.

Vorndam, V. and G. Kuno. 1997. Laboratory diagnosis of dengue virus infections, p.313-334. In D.J. Gubler and G. Kuno (ed.), Dengue and dengue hemorrhagic fever. CAB International.

Wallach, D., J. Bigda, and H. Engelmann. 1999. The tumor necrosis factor (TNF) family and related molecules,p. 51-84. In J. Theze (ed.), The cytokine network and immune functions. Oxford University Press, Oxford, U.K.

Wang, L., R.F. Chen, J.W. Liu, I.K. Lee, C.P. Lee, H.C. Kuo, S.K. Huang, and K.D. Yang. 2011. DC-SIGN (CD209) promoter-336 A/G polymorphism is associated with dengue hemorrhagic fever and correlated to DC-SIGN expression and immune augmentation. PLoS Neg Trop Dis. **5(1):**e934. doi:10.1371/journal.pntd.0000934.

Wang, S.M. and S. Devi Sekaran. 2010. Evaluation of a commercial SD dengue virus NS1 antigen capture enzyme-linked immunosorbent assay kit for early diagnosis of dengue virus infection. J. Clin. Microbiol. **48(8):**2793-2797.

Wang W.K., H.L. Chen, C.F. Yang, S.C. Hsieh, C.C. Juan, S.M. Chang, C.C. Yu, L.H. Lin, J.H. Huang, and C.C. King. 2006. Slower rates of clearance of viral load and virus-containing immune complexes in patients with dengue hemorrhagic fever. Clin Infect Dis. **43(8):**1023-30.

Wati, S., P. Li, C.J. Burrell, and J.M. Carr. 2007. Dengue virus (DV) replication in monocytes-derived macrophages is not affected by tumor necrosis factor alpha (TNF-α), and DV infection induces altered responsiveness to TNF-α stimulation. J. Virol. **81(18):**10161-10171.

Wearing, H.J. and P. Rohani. 2006. Ecological and immunological determinants of dengue epidemics. PNAS. **103(31):**11802-11807.

Webster, D.P., J. Farrar, and S. Rowland-Jones. 2009. Progress towards a dengue vaccine. Lancet Infect. Dis. **9:**678-687.

Webster, L.T. 1938. Japanese B encephalitis virus: its differentiation from St. Louis encephalitis virus and relationship to louping ill virus. J. Exp. Med. **67(4):**609-618.

Wetzler, L.M. and H.K. Guttormsen. 2004. B-lymphocyte activation and antibody production, p. 233-258. In G.B. Pier, J.B. Lyczak, and L.M. Wetzler (ed.), Immunology, infection, and immunity. ASM Press, Washington D.C.

White, L.A. 1987. Susceptibility of Aedes aegypti C6/36 cells to viral infection. J. Clin. Microbiol. **25(7):**1221-1224.

White, S.K., N.M. Iovine, L.C. Nickles, J.G. Morris, Jr., and J.A. Lednicky. 2017. Complete genome sequence of dengue virus type 2 from a resident of north-central Florida with locally transmitted dengue fever. Genome Announc. **5:e00782-17.** https://doi.org/10.1128/genomeA.00782-17.

Whitehead, S.S. and A.P. Durbin. 2010. Prospects and challenges for dengue virus vaccine development, p.221-237. In K.A. Hanley and S.C. Weaver (ed.), Frontiers in dengue virus research. Caister Academic Press, Norfolk, U.K.

Wichmann, O., K. Vannice, E.J. Asturias, E.J.A. Luna, I. Longini, A.L. Lopez, P.G. Smith, H. Tissera, I.K. Yoon, and J. Hombach. 2017. Live attenuated tetravalent dengue vaccines: the needs and

challenges of post-licensure evaluation of vaccine safety and effectiveness. Vaccine. **35:**5535-5542. http://dx/doi.org/10.1016/j.vaccine.2017.08.066.

Wilbar, C.L. 1947. Control of dengue in Hawaii. Am. J. Public Health Nations Health. **37(6):**663-674.

Winkler, G. 1988. Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. Virology. **162(1):**187-196.

Williams, K.L., S. Zompi, P.R. Beatty, and E. Harris. 2009. A mouse model for studying dengue virus pathogenesis and immune response. Ann. N.Y. Acad. Sci. **1171:**E12-E23.

Winskill, P., D.O. Carvalho, M.L. Capurro, L. Alphey, C.A. Donnelly, and A.R. McKemey. 2015. Dispersal of engineered male Aedes aegypti mosquitoes. PLoS Neg. Trop. Dis. **9(11):**e0004156. doi:10.1371/journal.pntd.0004156.

World Health Organization (WHO). 2015. Dengue and severe dengue. http://www.who.int/mediacentre/factsheets/fs117/en/.

World Health Organization (WHO). 2012. Dengue fever in Madeira, Portugal. http://www.who.int/csr/don/2012_10_17/en/#.

World Health Organization (WHO). 2011. Dengue NET. http://apps.who.int/globalatlas/DataQuery/default.asp.

World Health Organization (WHO). 2011. Dengue virus factsheet: http://www.who.int/mediacentre/factsheets/fs117/en/.

World Health Organization (WHO). 2009. Dengue: guidelines for diagnosis, treatment, prevention and control- new edition. WHO Press, Geneva, Switzerland.

Wuyts A, Struyf S, Proost P, van Damme J. 1999. Chemokines, p.125-45. In Theze J, editor. The Cytokine Network and Immune Functions. Oxford University Press, New York, New York.

Xie, X., Q.Y. Wang, H.Y. Xu, M. Qing, L. Kramer, Z. Yuan, and P.Y. Shi. 2011. Inhibition of dengue virus by targeting viral NS4B protein. J. Virol. **85(21):**11183-11195.

Yap, S.S.L., T. Nguyen-Khuong, P.M. Rudd, and S. Alonso. 2017. Dengue virus glycosylation: what do we know? Front. Microbiol. **8(1415).** doi:10.3389/fmicb.2017.01415.

Yauch, L.E. and S. Shresta. 2008. Mouse models of dengue virus infection and disease. Antiviral Research. **80:**87-93.

Ye, C., S. Abraham, H. Wu, P. Shankar, and N. Manjunath. 2011. Silencing early viral replication in macrophages and dendritic cells effectively suppresses flavivirus encephalitis. PLoS One. **6(3):**e17889. doi:10.1371/journal.pone.0017889.

Yen, Y.T., H.C. Chen, Y.D. Lin, C.C. Shieh, and B.A. Wu-Hsieh. 2008. Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species is key to hemorrhage development. J. Virol. **82(24):**12312-12324.

Yeo, A.S.L., N.A. Azhar, W. Yeow, C.C. Talbot, Jr., M.A. Khan, E.M. Shankar, A. Rathakrishnan, A. Azizan, S.M. Wang, S.K. Lee, M.Y. Fong, R. Manikam, and S.D. Sekaran. 2014. Lack of clinical manifestations in asymptomatic dengue infection is attributed to broad down-regulation and selective up-regulation of host defence response genes. PLoS One. **9(4):**e92240. doi:101371/journal.pone.0092240.

Yoshida C.F., C.D. Rouzerè, R.M. Nogueira, E. Lampe, M.A. Travassos-da-Rosa, B.O. Vanderborght, and H.G. Schatzmayr. 1992. Human antibodies to dengue and yellow fever do not react in diagnostic assays for hepatitis C virus. Braz J Med Biol Res. **25(11):**1131-5.

Yoshida, T., T. Omatsu, A. Saito, Y. Katakai, Y. Iwasaki, S. Iijima, T. Kurosawa, M. Hamano, S. Nakamura, T. Takasaki, Y. Yasutomi, I. Kurane, and H. Akari. 2011. CD16+ natural killer cells play a limited role against primary dengue virus infection in tamarins. Arch. Virol. **doi:10.1007/s00705-011-1178-6.**

Zamree, I., N. Drakes, A. Rohani, and H.L. Lee. 2005. Sensitivity of Aedes albopictus C6/36 cells line for the detection and infectivity titration of dengue virus. Trop. Biomed. **22(2):**217-219.

Zang, L., Q. Xu, Y. Ye, X. Li, Y. Liu, S.I. Tashiro, S. Onodera, and T. Ikejima. 2012. Autophagy enhanced phagocytosis of apoptotic cells by oridonin-treated human histiocytic lymphoma U937 cells. Arch. Biochem. Biophy. **518:**31-41. doi:10.1016/j.abb.2011.11.019.

Zargar, S., T.A. Wani, and S.K. Jain. 2010. Morphological changes in Vero cells postinfection with dengue virus type-2. Mic. Res. Tech. **doi:10.1002/jemt.20908.**

Zhang, B., J. Patel, M. Croyle, M.S. Diamond, and R.S. Klein. 2010. TNF-α-dependent regulation of CXCR3 expression modulates neuronal survival during West Nile encephalitis. J. Neuroimmunol. **224:**28-38.

Zhang, X. and D.M. Mosser. 2009. The functional heterogeneity of activated macrophages, p. 325-340. In D.G. Russell and S. Gordon (ed.), Phagocyte-pathogen interactions: macrophages and the host response to infection. ASM Press, Washington D.C.

Zheng, A., M. Umashankar, and M. Kielian. 2010. *In vitro* and *in vivo* studies identify important features of dengue virus pr-E protein interactions. PLoS Pathogens. **6(10):**e1001157. doi:10.1371/journal.ppat.1001157.

Zompi, S., B.H. Santich, P.R. Beatty, and E. Harris. 2011. Protection from secondary dengue virus infection in a mouse model reveals the role of serotype cross-reactive B and T cells. J. Immunol. **doi:10.4049/jimmunol.11021124.**

APPENDIX

A.1. Funding Information

The research detailed in this publication was supported jointly by Grant/Cooperative Agreement No. U38/CCU423095 from a grant funded by CDC/USDHHS and the Southeastern Center for Emerging Biologic Threats (SECEBT), Emory University (Atlanta, GA, USA). The contents described within are solely the responsibility of the Authors and do not necessarily represent the views of CDC/USDHHS or SECEBT.

Project Name: "Development of an *in-vitro* model system for assessing risk of progression to severe disease among Dengue, West Nile, and St. Louis Encephalitis infections"

PI Address/Contact: Azliyati Azizan, PhD, MSc
Assistant Professor
Department of Global Health
College of Public Health
University of South Florida
13201 Bruce B. Downs Blvd., MDC056
Tampa, Florida 33612-3805
aazizan@health.usf.edu

SECEBT Partner(s): Florida Department of Health
Lillian M. Stark, Ph.D., M.P.H., M.S.
Virology Administrator for the Bureau of Laboratories

Award amount: $48699

Additional funding was provided by the University of South Florida, College of Public Health.

Award amount: $1500
Type: Travel grant

Award amount: $1000
Type: Student Research Scholarship (SRS)

A.2. Amino Acid Codes

A (Ala) = alanine	H (His) = histidine	P (Pro) = proline	W (Trp) = tryptophan
C (Cys) = cysteine	I (Ile) = isoleucine	Q (Gln) = glutamine	Y (Tyr) = tyrosine
D (Asp) = aspartic acid	K (Lys) = lysine	R (Arg) = arginine	
E (Glu) = glutamic acid	L (Leu) = leucine	S (Ser) = serine	
F (Phe) = phenylalanine	M (Met) = methionine	T (Thr) = threonine	
G (Gly) = glycine	N (Asn) = asparagine	V (Val) = valine	

A.3. Nucleotide Codes

G = guanosine
A = adenosine
T = thymidine
C = cytidine